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The Journal of Biochemistry, Vol. 44, No. 6, 1957

STUDIES ON THE METABOLISM OF PARA-AMINOSALICYLIC ACID

I. PAPER CHROMATOGRAPHIC ANALYSIS OF HUMAN URINE

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(Received for publication, December 14, 1956)

Few reports have been done on the metabolic fate of *p*-aminosalicylic acid (PAS), in spite of its usual application in therapy. Previous studies have only revealed that the PAS given to human being is excreted in urine as unchanged, glycine conjugated and N-acetylated form (*1*, *2*). However, since it is natural to assume that benzene derivatives with different functional radicals such as PAS may undergo a complicated change *in vivo*, the author examined the metabolic products of PAS excreted in urine by means of paper chromatographic analysis and found at least nine different substances. While the experiment was in progress, Kawamata and Kashiwagi (*3*) reported that unchanged PAS, a glycine conjugate, N-acetyl PAS, N-acetyl PAS glucuronide and a glutamine conjugate were found in the urine of the man administered PAS. In the author's experiment, however, unchanged PAS, N-acetylated PAS, *p*-aminosalicyluric acid, a sulfate conjugate and five different glucuronides were detected in the urine.

In this paper, the characterization of the metablites of PAS is discussed. The further investigations of the glucuronides will be reported in the future (*4*).

EXPERIMENTAL

Material—A dose of 5 g. of PAS sodium or calcium salt was administered orally to man and the urine excreted from 1 to 6 hours was collected. The urine was concentrated to one tenth volume or less under the reduced pressure below 50°, and then, about 2 volumes of 96 per cent ethanol was added. The precipitate was centrifuged off and discarded.

Paper Chromatography—The remaining desalted urine was applied on Toyo filter paper No. 50 or No. 51 and the chromatograms were run in *n*-butanol-acetic acid-water (4:1:5) for about 18 hours and in methanol-benzene-*n*-butanol-water (2:1:1:1)

for about 10 hours. When the ultraviolet absorption curve was required, eluate of the spot was extracted with purified ether to remove impurities.

Following three sprays were used for the localization of spots.

(*1*) *Bratton-Marchall's Reagents:*

(a) Diazotizing solution. Equal parts of ice cold 1*N* HCl and 0.1 per cent sodium nitrite were mixed just before use.

(b) Ammonium sulfamate. 0.5 per cent aqueous solution.

(c) N-Naphthylethylendiamine hydrochloride. 0.1 per cent aqueous solution.

The reagents were cooled priviously in the ice box and the chromatograms were sprayed with sufficient amounts of above solutions successively in a cold room.

(*2*) *Ehrlich's Reagent:* On part of 5 per cent dimethyl aminobenzaldehyde in methanol and 3 parts of concentrated HCl were mixed.

(*3*) *Ferric Chloride:* 2 per cent aqueous solution.

Synthesis of N-acetyl PAS—To about 0.5 g. of PAS dissolved in 10 ml. of 2 *N* NaOH, 3 ml. of acetic anhydride was added under cooling. The white crystals obtained were recrystallized three times from water (m.p. 223–4°). It gave a purple color with ferric chloride. Bratton-Marshall's reaction was positive only after hydrolysis with diluted alkali for a few minutes at 100°, and PAS liberated was identified by paper chromatography. The substance developed a delayed color reaction of intensive yellow a few days after spraying with Ehrlich's reagent.

RESULTS

The Metabolic Products of PAS—Nine spots which originated from PAS were obtained with two-dimensional paper chromatograms run in *n*-butanol-acetic acid-water (4 :1 :5) and methanol-benzene-*n*-butanol-water (2 :1 :1 :1) as shown in Fig. 1. These spots were conveniently numbered in order of R_f value on paper with butanol-acetic acid-water. Besides these spots there appeared a faint blue spot with Bratton-Marshall's reagents at R_f 0.35 (in butanol-acetic acid-water) and a diffuse spot of faint yellow color with Ehrlich's reagent at R_f 0.40 where it almost covered the spot No. 5. Those two spots were not specific for the urine containing metabolites of PAS. The latter spot was demonstrated to be urea, whereas the nature of the former spot remained unclarified.

To test the hydrolyzability of each substance the desalted urine was hydrolyzed in 1 *N* sulfuric acid for 15 minutes at 100°, neutralized with barium hydroxide to phenolphthalein, and, after removal of barium sulfate by centrifugation, submitted to paper chromatography. Alkali hydrolysis was carried out in 1 *N* KOH, the hydrolysate was neutralized with perchloric acid and the resultant precipitate of potassium perchlorate was centrifuged off. By acid hydrolysis (Fig. 2) the spots No.

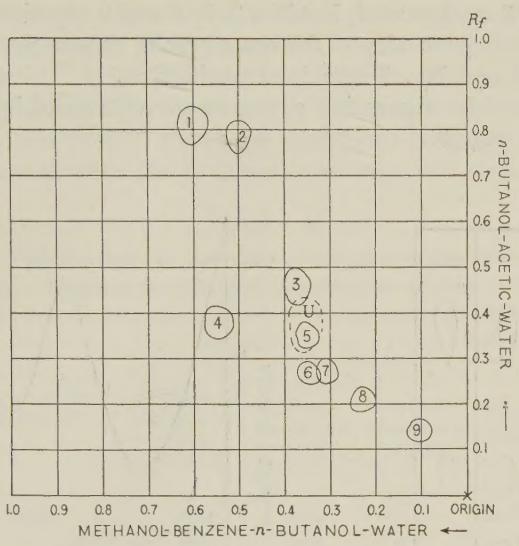

FIG. 1. Chromatogram of derivatives of PAS in human urine after ingestion of PAS. Solvents were *n*-butanol-acetic acid-water (4:1:5), followed by methanol-benzene-*n*-butanol-water (2:1:1:1).

Ehrlich's reagent was employed for spraying. U represents the spot of urea.

TABLE I

R_f Values and Intensity of Spots Illustrated in Fig. 1

Spot No.	R_f	Name of substances	Intenstity of spots		
			A	B	C
1–2	0.80	N-acetyl PAS[1] and PAS	++	++	++
3	0.46	*p*-Aminosalicyluric acid	++	++	++
4	0.38	Ester glucuronide of N-acetyl PAS	+	−	−
5	0.33	Ester glucuronide of N-acetyl PAS	+	−	−
6	0.28	Ester glucuronide of PAS	+	−	+
7	0.28	Sulfate conjugate[2]	−	−	−
8	0.19	Unknown glucuronide	+	−	+
9	0.14	Ether glucuronide of PAS	+	−	+

1) When the chromatogram was run in butanol-acetic acid-water, the spots of PAS and N-acetyl PAS were amalgamated usually.

2) An ethereal sulfate was not revealed on this sample perhaps because it was destroyed while the ruine was concentrated.

1, 4, 5, 7 and 8 disappeared, but No. 2, 3, 6 and 9 remained unchanged. Alkali hydrolysis gave almost the same results except that the spot No. 6 disappeared and No. 7 remained unchanged.

The ultraviolet-absorption curves of the eluates of individual spots are shown in Fig. 3.

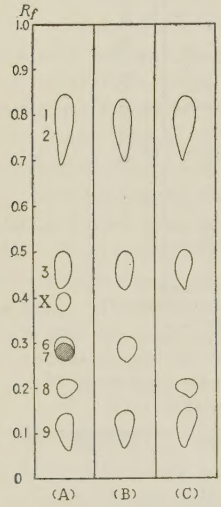

FIG. 2. Chromatograms of derivatives of PAS in human urine before (A) and after hydrolysis in 1*N* sulfuric acid for 15 minutes at 100° or in 1*N* potassium hydroxyde for 15 minutes at 100° (C). The solvent was butanol-acetic acid-water (4:1:5). The spots were colored with Bratton-Marshall's reagents. X represents an unspecific blue spot.

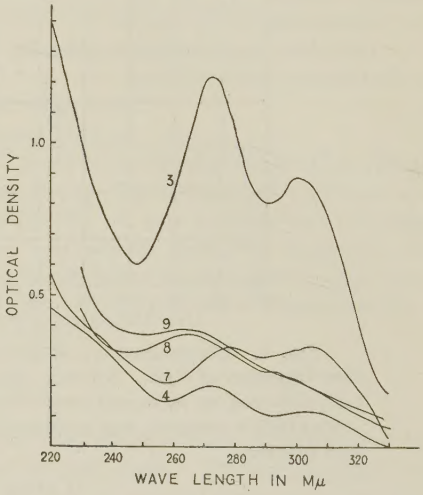

FIG. 3. Ultra-violet absorption curves of the eluates at pH 7.0 from the chromatogram.

Characterization and Identification of the Individual Spots—The results of the following experiments are summarized in Table II.

Spots No. 1 and 2 were not separable on one-dimensional chromatogram run in butanol-acetic acid-water. After eluting the combined spots from the paper with water, the eluate was rechromatographed with

a solvent of benzene-acetic acid-water (2 :1 :1). Two spots were clearly demonstrated by spraying with ferric chloride as well as with Ehrlich's reagent. The spot with an R_f value of 0.37 gave an intensive red color with Bratton-Marshall's reagents, whereas the spot with an R_f value of 0.08 did not. Furthermore, the latter spot was not visible after acid or alkali hydrolysis. From the agreement of the R_f values and of the color reactions with the authentic specimen, the spots No. 1 and 2

TABLE II

Characteristics of Each Spot on the Chromatogram of Human Urine after Oral Administration of PAS

Spot No.	Supposed name of compound	R_f Value		Color reaction		
		In butanol-acetic acid-water (4:1:5)	In methanol-benzen-butanol-water (2:1:1:1)	Bratton-Marshall's reagents	Ferric chloride	Ehrlich's reagent
1	N-acetyl PAS	0.82	0.60	–	Br–V	Y(delayed)
2	PAS	0.79	0.50	R	Br–V	Y
3	*p*-Aminosalicyluric acid	0.46	0.38	R–V	Br–V	Y
4	Ester glucuronide of N-acetyl PAS	0.38	0.55	–	Br–V	Y(delayed)
5	Ester glucuronide of N-acetyl PAS	0.33	0.35	–	–	Y(delayed)
6	Ester glucuronide of PAS	0.28	0.35	R	Br–V	Y
7	Sulfate conjugate	0.28	0.32	Bl–V	Or–Br	Y
8	Unknown glucuronide	0.19	0.22	R	Br–V	Y
9	Ether glucuronide of PAS	0.14	0.12	R–V	–	Y

In this table, the following abbreviations are used: blue (Bl), brown (Br), orange (Or), red (R), violet (V), yellow (Y).

were identified with N-acetyl PAS and uncombined PAS, respectively. Some investigators have already reported that PAS and N-acetyl PAS are excreted in urine after the ingestion of PAS. (*1, 2, 3*).

The spot No. 3 was proved to be *p*-aminosalicyluric acid as follows. Because urine contained various free amino acids which interfered the detection of any conjugated amino acid, it was necessary to destroy the

free amino acids beforehand. A relatively large quantity of the material was applied on the paper in a stripe and a one-dimensional chromatogram was developed with butanol-acetic acid-water (4 :1 :5). After drying, the paper was sprayed with Bratton-Marshall's reagents. The band of No. 3 which showed an intensive red violet color was cut out and eluted with hot water. The eluate was allowed to react with the mixture of 1 *N* HCl and 0.1 per cent sodium nitrite for a few hours at room temperature until no more ninhydrin reaction occurred. After removal of the remaining nitrous acid by adding excess of urea, the solution was refluxed with two volumes of concentrated hydrochloric acid for more than 5 hours to hydrolyze the conjugate and evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of water and developed with three solvent systems, *n*-butanol-acetic acid-water (4 :1 :5), phenol saturated with water and *n*-butanol saturated with 3 per cent ammonia, along with authentic amino acids.

A spot appeared on each chromatogram by spraying with 0.1 per cent ninhydrin, the R_f values bing 0.15, 0.40 and 0.04, respectively, in agreement with the authentic sample of glycine.

As seen in Fig. 2, the substance of spot No. 3 was considerably resistent to acid and alkali hydrolysis, and was not affected by the action of crude glucuronidase prepared from mouse liver (*4*). Furthermore, this spot showed a reaction with ferric chloride indicating the existence of free phenolic hydroxy radical. These facts lead to the above conclusion that this spot was of *p*-aminosalicyluric acid.

No amino acid was detected from the spots No. 4–9 by the same procedure.

The spot No. 7 which overlapped with the spot No. 6 on paper with butanol-acetic-acid-water but could be distinguished from the latter by its blue-violet color by spraying with Bratton-Marshall's reagents was demonstrated to be a sulfate conjugate as described below. By circular paper chromatography these two spots were separated more distinctly.

For the detection of sulfate conjugate, each colored spot obtained by Bratton-Marshall's test was cut out, eluted with hot water, concentrated to about 0.1 ml. and, after hydrolysis in diluted HCl, mixed with one drop of 0.1 per cent barium chloride solution. Only the spot No. 7 gave microcrystals of barium sulfate under a microscope.

Moreover, radioactive indicator was used for the detection of sulfate conjugate. About 100 microcuries of carrier-free S^{35}-sulfate was administered together with 0.3 g. of PAS to a dog weighing about 4 kg.

by intraperitoneal injection, and the urine was collected for 24 hours. After standing a few minutes with two volumes of 95 per cent ethanol, the precipitated salt was centrifuged off. The supernatant was cautiously concentrated *in vacuo* to the original volume, placed on the paper three times with the aid of small capillary and a paper chromatogram was developed. The paper was dried, sprayed with Bratton-Marshall's reagents, dried again and kept in contact with Fuji No-Screen Film in a cassette for about 2 weeks. There appeared a spot on the radioautogram which agreed exactly with the blue-violet spot No. 7 of Bratton-Marshall's reaction. The spot was specific for the urine after administration of PAS, because no spot was observed on the radioautogram in case of injection of the same dose of radioactive sulfate omitting the administration of PAS. This substance was so unstable that it was lost by decomposition if the desalted supernatant was evaporated to dryness before the chromatography.

It was difficult to isolate the sulfate conjugate in a sufficient amount, so that it remains undecided whether it is a conjugate of PAS or its derivative such as oxidized PAS.

To detect glucuronide, each spot visualized by Bratton-Marshall's reaction was cut out exactly along the boundary, eluted with hot water until no color remained and the eluate which was concentrated *in vacuo* was examined by naphthoresorcinol test. The eluates of spots No. 6, 8 and 9 gave a positive reaction of glucuronic acid. Spot No. 4 and No. 5 were located in the following way. The sample was applied on the sheet of paper in a stripe and submitted to chromatography. The paper was sprayed with Ehrlich's reagent. The eluates of the areas corresponding to No. 4 and No. 5 were tested by naphthoresorcinol reaction. They gave also a positive reaction of glucuronic acid. Since PAS possesses one hydroxyl and one carboxyl group, both of which can be conjugated with glucuronic acid, and, furthermore, the amino group can be acetylated, there are four possible glucronides in all. It is well established that ether glucuronide of aromatic compounds is resistant against alkali hydrolysis, while ester glucuronide is very easily hydrolysed by alkali treatment. The spot No. 6 which was labile to alkali and gave a positive ferric chloride reaction was considered to be ester glucuronide. The sopt No. 9 which was resistant against alkali hydrolysis and relatively stable to acid treatment, giving no color reaction with ferric chloride was considered to be ether glucronide (Fig. 2). That both of the spots gave a positive Bratton-Marshall's reaction and prompt coloration with Ehrlich's reagent indicates the

amino group ramins free. The spots No. 4 and 5 are probably due to the two types of N-acetyl-PAS-glucuronides.

DISCUSSION

Kawamata and Kashiwagi (*3*) reported the presence of *p*-aminosalicylylglutamine among the metabolic products of PAS in human urine. To ascertain their observation the present author examined each of the spots visualized by Ehrlich's reaction as described above. However, only amino acid discovered by this procedure was glycine. Kawamata and his collaborators described that the R_f value of *p*-aminosalicylylglutamine was 0.38, when run in butanol-acetic acid-water (4 :-1 :2). Since the R_f values of the spots of PAS derivatives obtained with this solvent are somewhat lower than the values obtained by the solvent mixture of 4 :1 :5, the spot No. 6 or 7 might correspond to the spot with R_f vlue of 0.38 of Kawamata and his collaborators. However, no amino acid could be detected after hydrolysis in the eluate from the spots No. 6 and 7. Furthermore, it is strange enough that Kawamata and Hiratani (*5*) have detected the glutamine conjugate in rat urine, because no animal has been known undoubtedly to excrete glutamine conjugate of aromatic acid except human being and chimpanzee (*6*). It seems to need further investigation before concluding the production of glutamine conjugate as detoxication product of PAS.

Another possible amino acid which may combine with PAS or its metabolite is cysteine, since nitrophenylmercapturic acid has been discovered in the rabbit urine after administration of nitrophenol (*7*). An attempt was made to find out the acetylcysteine conjugate, but no proof of its existence was obtained.

The spot No. 7 seems most likely to be sulfate of PAS. The development of a faint but distinct color by spraying with ferric chloride may be explained by highly unstable nature of PAS sulfate which is hydrolyzed spontaneously on the paper before treating with ferric chloride solution. But the possibility that the substance is the sulfate conjugate of an oxidation product of PAS which possesses an additional uncombined hydroxyl group can not be ruled out.

There appeared five spots corresponding to glucuronides, characterization and identification of which will be discussed in detail in the future.

SUMMARY

After administration of *p*-aminosalicylate to human beings, the excreted urines contained nine compounds originating from PAS, *i.e.*, unchanged PAS, N-acetyl-PAS, *p*-aminosalicyluric acid, a sulfate conjugate, and five glucuronides, including ether type and ester type glucuronides of PAS and N-acetyl PAS. Glutamine conjugate of PAS could not be detected.

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CONTRACTILE PROTEINS FROM ADDUCTORS OF PECTEN

IV. CONSTITUTION OF MYOSIN B

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(Received for publication, Junuary 16, 1957)

In the previous papers (*1*, *2*) on the biochemical properties of myosin B from the fast and the slow adductors of pecten, it was found that the adenosine triphosphatase (ATPase) activity (per g. of protein) of the fast adductor myosin B is 3 times as high as that of slow adductor, but the Michaelis constants of the two myosin B are identical. It was also observed that the functional unit weight of the slow adductor myosin B, *i.e.*, the reciprocal of the minimum ATP necessary for the maximum response, is about 3 times as high as the value for the fast adductor.

As is well known (*3*), the physico-chemical properties, *i.e.*, the viscosity, the light-scattering, *etc.*, of the actomyosin solution are changed by the addition of adenosine triphosphate (ATP), but the properties of the myosin solution do not change by the ATP addition. Furthermore, myosin B may be regarded as a mixture of myosin and actomyosin. Therefore, in order to extract the physical meaning from the above mentioned correlation between ATPase activities and the functional unit weights of the two myosin B, it is necessary to demonstrate that the minimum quantity of ATP for the maximum response is determined by the quantity of myosin contained in the myosin B preparations and is not dependent on the quantity of actin.

For this purpose, four kinds of fast adductor myosin B were prepared, differing greatly in their extraction time. Their protein compositions were determined by salting-out analysis and each functional unit weight was estimated by the light-scattering method (*4*).

All experiments on the change of light-scattering reported in the previous paper (*2*) were performed only in the presence of Mg^{++}. Now, the relation between the increased concentration of ATP and the response of myosin B were also investigated in other ionic environments and their results will be reported in this paper.

EXPERIMENTAL

Four kinds of myosin B were obtained by extracting homogenates of the fast adductor muscles of *Pecten yessoensis* for suitable times (3 or 10 minutes, 30 minutes, 3 hours and 24 hours) with the Weber-Edsall solution, and purified by twice repeating the usual "dilution-precipitation method" (*3*). These myosin B showed weak adenylate-kinase activity (*2*) and no adenylic acid deaminase activity (*5*).

ATP was prepared from fresh rabbit skeletal muscles by Kerr's method modified by A. Szent-Györgyi (*3*) and was used as a potassium salt throughout the experiments. The purity of ATP was tested by chromatographic analysis (*6*) and found to be 92 per cent. The most frequent impurity was adenosine diphosphate; others which were found as trace amounts, included adenylic acid and inosinic acid. Following this results, a correction factor of 0.94 was used for computation of the ATP concentration from 7 minutes-P.

Salting-out analysis of myosin B was carried out under experimental conditions almost identical with those used by Snellman and Tenow (*7*) in their experiments with uterine muscle protein. In the experiments twice recrystallized ammonium sulfate was used and both KH_2PO_4 and K_2HPO_4 were guaranteed grade. Stock solutions of phosphate buffer (0.6 *M* potassium ion and 0.1 *M* phosphate ions; pH 6.9) with and without saturating amount of ammonium sulfate were prepared at 0°. Combining these in calculated proportions, a series of salt solutions with varying concentrations of ammonium sulfate and constant concentrations of buffer salts were obtained. To 4.5 ml. of these solutions, 1.5 ml. of each of myosin B (2–3 mg. of protein per ml.; 0.6 *M* KCl) were added and the mixture was allowed to stand for 20 hours at 0°. The solution was filtered through Toyo No. 2 filter paper and the protein in the filtrate was determined by measuring the extinction at 280 mμ with a Shimazu ultraviolet photospectrometer.

Viscosity was determined by a viscosimeter of the Ostwald type at 14±0.1°. The flow-time of myosin B was about 100 seconds. The intensity of light scattered at a 90° angle was measured with an electromultiplier-μ ammeter system, as described in our previous paper (*4*). The pH was determined by a Beckman-G-type pH-meter. Nitrogen contents were determined by the micro-Kjeldahl method and the contents of protein were calculated by using a factor of 6.

RESULTS

I. The Change of Viscosity

The sensitivity of the viscosity drop of the myosin B solution after the ATP addition is defined as follows (*8*):

$$\text{ATP sensitivity} = \frac{Z\eta_{\text{rel}} - Z\eta_{\text{ATP}}}{Z\eta_{\text{ATP}}} \times 100$$

where $Z\eta_{\text{rel}}$ and $Z\eta_{\text{ATP}}$ represent the logarithms, respectively, of the relative viscosities of myosin B (0.6 *M* KCl) solution before and after

the addition of ATP. ATP sensitivities of three kinds of myosin B's extracted for different periods—10 minutes, 3 hours and 24 hours—were measured and the results are tabulated in Table I. There are some variations from one preparation to another, but the value for myosin B extracted for the short time (10 minutes) is considerably lower than the one extracted for the long time (3 or 24 hours). ATP sensitivity changed only slightly by the extension of extraction time from 30 minutes to 24 hours.

II. Salting-Out Analysis of Myosin B

Salting-out analyses by ammonium sulfate were carried out in order to investigate the composition of the three kinds of myosin B which differ in their extraction times. The measurements of the extinctions were carried out at room temperature, and, at a concentration ranging

TABLE I

Dependence of ATP Sensitivity of Myosin B on the Extraction Time

Myosin B extracted during	10 min.	3 hrs.	24 hrs.	
Concentration (*mg./ml.*)	2.8	3.2	2.1	2.6
η_{rel}	2.10	3.28	1.88	3.64
η_{ATP}	1.63	1.67	1.35	1.90
ATP sensitivity (%)	52	120	116	103

from 25 to 32 per cent ammonium sulfate, the proteins remaining in the filtrate were found to become turbid sooner or later, because solubility of actomyosin is decreased with rise of temperature (*9*).

In Fig. 1 the optical density (S) of the filtrate is plotted against the concentration (C) of ammonium sulfate. Also the differential extinction ($\Delta S/\Delta C$) as a function of concentration has been computed. The latter diagram is also given in Fig. 2. It is evident from the figures that pecten myosin B consists of two components: the A component which precipitates between 37–44 per cent and the B component which precipitates between 28–37 per cent. The precipitation range of the A component remained the same, but the precipitation range of the B component changed considerably with the extension of extraction time, that is, the concentrations for the maximum $\Delta S/\Delta C$ were found to be

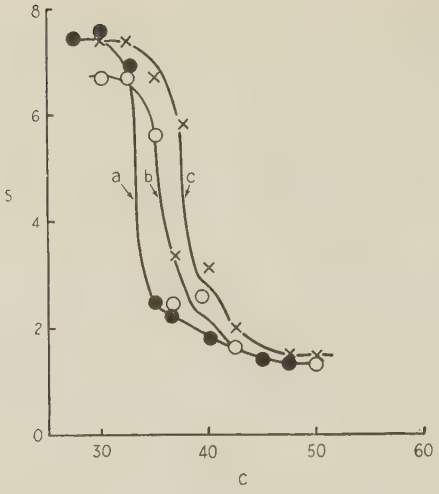

FIG. 1. Salting-out curves of the myosin B extracted during variable times. S, extinction at 280 mμ (1 g. of N/litre, 1 cm. light path); C, percentage volume of saturated ammonium sulfate. ●, myosin B extracted during 24 hours (0.48 mg. of protein/ml.); ○, myosin B extracted during 30 minutes (0.45 mg. of protein/ml.); ×, myosin B extracted during 10 minutes (0.4 mg. of protein/ml.).

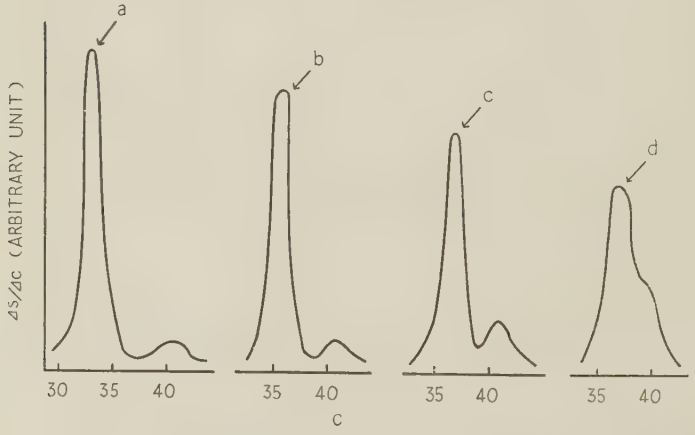

FIG. 2. Salting-out maps taken from the salting-out curves (Fig. 1). S, extinction at 280 mμ (1 g. of N/litre, 1 cm light path); C, percentage volume of saturated ammonium sulfate. Extinction time of each myosin B; a, 24 hours; b, 30 minutes; c, 10 minutes; d, 3 minutes.

37, 35–36 and 32–33 per cent for myosin B extracted respectively in 10 minutes, 30 minutes and 24 hours. Quantitative data for the two components cannot be obtained from the curves without a knowledge of the specific extinction coefficient of the components, but it may be deduced from the figures that the A component decreased with extraction time, while the B component behaved in the opposite manner.

Under experimental conditions similar to ours, the precipitation ranges of actomyosin and myosin from vertebrate muscles are 28–32 and 33–45 per cent, respectively (*7*, *10*). As is well known (*3*), the yield of actomyosin increases with extraction time and the yield of myosin

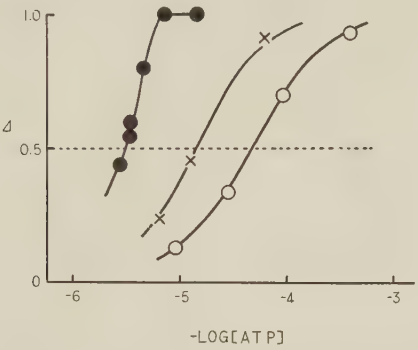

FIG. 3. Effects of Ca++ and Mg++ on the relationship between grade (Δ) of the drop of light scattering and the concentration of ATP added. ●, $MgCl_2$ 2.5 mM; ○, $CaCl_2$ 2.5 mM; ×, no $MgCl_2$ and $CaCl_2$.

decreases with time. Therefore, there can be little doubt that the A component is myosin and the B component is actomyosin.

III. Effects of Ions on the Combination of ATP with Myosin B

By the addition of ATP to myosin B solution (0.6 *M* KCl), marked reduction in the light-scattering was observed. Let us denote the grade of the drop (Δ) as follows;

$$\Delta = \frac{I_O - I_S}{I_O - I_\infty}$$

where I_O and I_S represent the intensities of light scattered at 90° before and after the addition of a certain amount of ATP, respectively. I_∞ stands for the light-scattering with sufficiently large quantity of ATP. The quantity Δ, then, is indicative of the ratio of the amount of protein molecules deformed to the total in the solution studied (*11*, *12*).

By the addition of Ca^{++} or Mg^{++} the amount of $I_O - I_\infty$ remains unchanged, but the quantity of ATP necessary for the reduction of light-scattering is changed greatly. As shown in Fig. 3, in the absence of Mg^{++}, the relation between Δ and logarithms of the concentration of added ATP ($[\Sigma\ S]$) is represented as a sygmoid curve of the first order. The ϕ-values, *i.e.*, the dissociation constants of the combination of ATP with myosin B, were found to be 2.2×10^{-5} and $6.3 \times 10^{-5}\ M$ in the absence of and also in the presence of Ca^{++}, respectively.

On the other hand, the addition of Mg^{++} reduced the ϕ-value

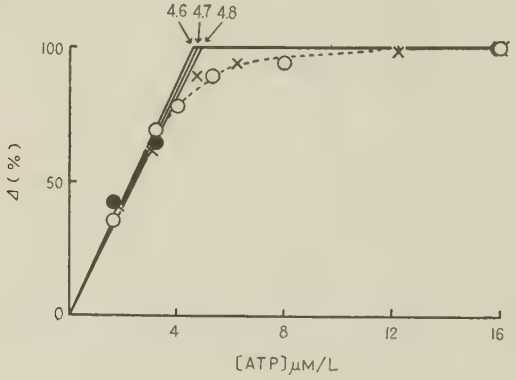

Fig. 4. Combination of ATP with myosin B in the presence of Mg^{++} (2.5 mM). Extraction time of each myosin B: ×, myosin B extracted during 24 hours. (1.87 mg./ml.); ●, myosin B extracted during 30 minutes (1.78 mg./ml.); ○, myosin B extracted during 10 minutes (1.57 mg./ml.). pH 6.4, 13°.

extremely, and as the concentration of myosin B used in the experiments was considerably high (about 2 mg. of protein/ml.), the ϕ-value became much lower than the concentration of the ATP binding site of myosin B (about 5 μM, see below). Then, Δ was proportional to the ATP concentrations within the low quantity. Further, over the critical concentration of ATP, Δ became constant (Fig. 4). From the critical value of ATP, the amount of myosin B combined with 1 g. mole, *i.e.*, the functional unit weight of myosin B can be calculated.

IV. Functional Unit Weights of Various Myosin B's

The functional unit weights of myosin B's extracted for 10 minutes, 30 minutes and 24 hours, were determined by the above mentioned method, and the results are summarized in Table II. The value in-

creased slightly with extraction time.

DISCUSSION

As stated above, an extension of extraction time from 30 minutes to 24 hours resulted in a slight change of the ATP sensitivity of the protein solution. Similar situations were already observed in the extraction of myosin B from locust muscle by Gilmour and Calaby (*13*). This indicates that the rapidity of extraction of actin from invertebrate muscle is quite different from the situation in rabbit muscle, in which a 20 minutes extract consisted of almost pure myosin (*14*).

Two alternative suppositions are now prevalent as to the constitution of myosin B. The one adopted by Morales *et al.* (*15*), states that myosin B is formed in extraction by self-aggregation, *i.e.*, it is mainly an aggregate of myosin A. The other adopted by many workers in this

TABLE II

Dependence of Functional Unit Weight of Myosin B on the Extraction Time

Myosin B extracted during	*min.* 10	*min.* 30	*hrs.* 24
Concentration (*mg./ml.*)	1.57	1.78	1.87
Critical concentration of ATP ($\mu M/l.$)	4.6	4.8	4.7
Functional unit wight (10^{-5} *g.*)	3.4	3.7	4.0

field (*3*), hypothesizes that myosin B is a mixture of myosin and actomyosin, and actomyosin is the stoichiometric complex of myosin and actin. Results from the salting-out analysis of pecten myosin B are, on the one hand, apparently in conflict with Morales' assumption, because, as stated above, myosin B constitutes myosin (the A component) and actomyosin (the B component). But, on the other hand, actomyosin cannot be the stoichiometric complex of actin and myosin, because the precipitation range of actomyosin is not constant and varies considerably with increasing extraction time.

As is well known (*8*), it is impossible to separate myosin B quantitatively into pure actin and myosin. Moreover, the specific extinction coefficient of invertebrate myosin and actin are not known. Therefore, it is impossible to determine amounts of actin included in various myosin B's. However, we have seen that (1) with increasing the extraction

time, the precipitating range of the B component decreased from that of pure myosin to that of actomyosin contaminated with a high proportion of actin, and, further, (2) myosin extracted during a long time showed higher ATP sensitivity than did for myosin B extracted for short time. These results indicate that actin proportion in myosin B increases with extraction time. According to A. G. Szent-Györgyi (*16*), ATP sensitivity is approximately proportional to actin contents. Therefore, it may be said that the content of actin in myosin B extracted during 30 minutes and 24 hours is about twice as high as its content in myosin B extracted during 10 minutes.

Nevertheless, the quantity of ATP necessary for the change of light-scattering (in the presence of Mg^{++}) decreases, *i.e.*, the functional unit weight increases slightly with extraction time. When we consider that in myosin B the quantity of actin is much lower than that of myosin (probably only 1/4, at most, of myosin), these results can best be accounted for by an assumption that the physico-chemical properties of myosin B are changed by the combination with ATP at the active site of the *myosin* mocecule, and so, the quantity of ATP necessary for the changes is proportional to the quantity of myosin.

As described above, the combination of ATP with myosin B is represented by a dissociation curve, and the combination is strengthened by Mg^{++} and weakened by Ca^{++}. Similar results have already been obtained with the myosin B of rabbit skeletal muscle, but in this case Ca^{++} strengthened the combination slightly (*11*).

SUMMARY

1. Four kinds of myosin B of various extraction time—3 or 10 minutes, 30 minutes, 3 hours and 24 hours—were prepared from the fast adductor muscle of *Pecten yessoensis*.

2. By extension of extraction time from 10 minutes to 30 minutes or to 24 hours, ATP sentitivity increases twice.

3. Composition of four kinds of myosin B was investigated by salting-out analysis, and it was found that they consist mostly of myosin and actomyosin.

4. Their functional unit weights increase slightly with extraction time.

5. From these results it was concluded that the properties of myosin B are changed by the combination of ATP with the active site

of the myosin molecule.

6. Mg^{++} strengthens the combination of ATP at the active site, and Ca^{++} weakens the combination.

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CONTRACTILE PROTEINS FROM ADDUCTORS OF PECTEN

V. PROCESS OF RECOVERY FROM DROP IN LIGHT SCATTERING CAUSED BY ADENOSINE TRIPHOSPHATE[1]

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As well known, when ATP is added to actomyosin solution (0.6 *M* KCl), there is a decrease in the intensity of scattered light; following this is a period during which the reduced intensity remains constant; finally, when ATP is broken down by myosin ATPase, the light scattering intensity returns to the original value (*1*, *2*). Studies from the viewpoint of transient kinetics on the decrease of light scattering have been published by Tonomura and coworkers (*3-5*). The change of the size and shape of actomyosin particles after the addition of ATP has been elucidated by Blum and Morales (*2*) and Gergely (*6*), using the Zimm method.

The viscosity of actomyosin solution is also decreased by the addition of ATP (*7*). Csapó (*8*) has demonstrated that the period during which the reduced viscosity remains constant is almost proportional to the quantity of ATP added. However, the mechanism of the recovery process has not yet been made clear except for the fact that the recovery begins after the ATP content has been decreased to some extent by the action of ATPase. The fall in viscosity and light scattering also are effected by Mg^{++}-PP, but in this case there is no recovery from those effects, because PP is not hydrolyzed by myosin (*9*, *10*).

In previous papers (*11*, *12*) in which the present authors had a share it was reported that a small quantity of arginine, Mg^{++} and a protein, probably arginine kinase, are essential factors for the recovery

1) Following contractions are used in this paper: adenosine triphosphate, ATP; adenosinetriphosphatase, ATPase; adenosine diphosphate, ADP; adenylic acid, AMP; inorganic pyrophosphate, PP; pyrophosphatase, PPase; inorganic orthophosphate, P.

process of pecten myosin B and that when arginine is added in the early stage of the recovery, the viscosity drops again. In this paper, a study is presented on the recovery process of myosin B from the fast adductor of pecten (sufficient quantities of the cofactors necessary to the recovery being involved), mainly tracing the change by the light scattering method. The following results were obtained; (1) when concentrated myosin B solution is used, a labile structure seems to be set up at the early stage of recovery which is broken easily by arginine; (2) from the quantitative analysis of ATP by the Cohn-Carter method, it was demonstrated that in the presence of Mg^{++}, the light scattering of the reaction mixture begins to rise, after the quantity of ATP has been reduced to a point considerably less than the minimum one necessary to the maximum drop of scattered light; and (3) the reduced viscosity caused by Mg^{++}-PP recovers quite easily by the subsequent addition of PPase.

EXPERIMENTAL

ATP was prepared from fresh muscle of rabbit as a barium salt by Kerr's method (*13*) and was converted for use as a potassium salt with further purification on ion exchange resins. Its purity was about 93 95 per cent. Myosin B was obtained by extracting homogenates of the fast adductor muscle of *Pecten yessoensis* for 24 hours at 0° with Weber-Edsall's solution and purified by a twice repetition of the usual "dilution-precipitation" method (*14*). The obtained myosin B preparations were contaminated with an appreciable amount of adenylate kinase (see later). PPase was isolated from fresh baker's yeast (purchased from the Nippon Beet Sugar Manufacturing Co. Ltd., Tokachi-Shimizu Factory) by Kunitz' method (*15*) and purified to the step 3 of his direction. The specific activity of the preparation was about 0.6. Arginine, creatine, lysine, glycocyamine and PP were commercial products. The solutions of these reagents were adjusted to pH 7.0 by the addition of HCl or $NaHCO_3$.

Equipment for the measurement of light scattering was modified from the one reported previously (*3*) and are shown in Fig. 1; the electric circuit for photomultiplier RCA 931A was assembled according to Wada (*16*) with slight modifications. Pyrex glass cell of $2\times2\times5$ cm. was usually employed in which 10 ml. of myosin B solution was injected; the intensity of the scattered light was controlled by an iris stop and a slit so that the reading of the μ-ammeter for myosin B solution became 95 μA. Two-tenths ml. of ATP or other substances was added to myosin B solution with a pipette whose tip was cut, while the solution was stirred vigorously with a small glass spatula. An Ostwald type viscosimeter was used of which flow time was about 40 seconds for pure water.

Quantitative analysis of nucleotides was effected by chromatographic analysis on a column (0.7 cm. in diameter, 3 cm. in height) of Dowex 1 (200–400 mesh, 10 per cent cross linkage) in the chloride cycle according to Cohn and Carter (*17*). Adsorption

and elution was performed at 0–5°. Extinction of nucleotides was measured by a Shimazu ultraviolet spectrophotometer (at 260 mμ) with 1 cm. light path. Concentration of adenylic derivatives were computed, using a factor of 14,200 for the molar extinction coefficient.

The concentration of myosin B was determined by the micro Kjeldahl method, a factor of 6 being used for computation. The pH value was measured with a Beckman type G pH-meter. All experiments were carried out at 13° and pH 6.7 without using a buffer solution.

RESULTS

I. Labile Structure Built in Recovery Process

As indicated in Fig. 2, under the same experimental conditions,

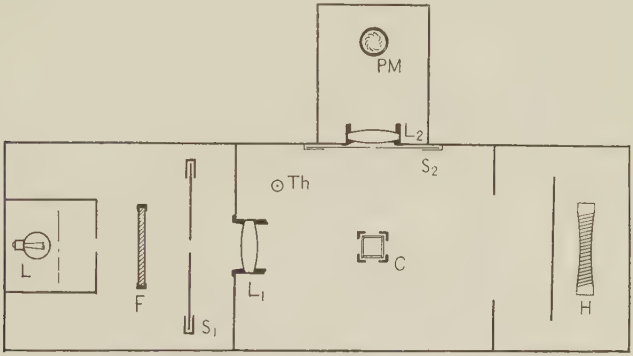


FIG. 1. Apparatus for the light scattering measurement.
L: lamp (6v., 35w.) with battery, F: interference filter, S_1: iris stop, S_2: slit, L_1, L_2: lens, Th: thermometer, H: heater, C: cubett, PM: photomultiplier RCA 931A.

the viscosity and the light scattering changes induced by the addition of ATP follow almost the same time course. Therefore, in the following experiments a more favorable light scattering measurement, than the viscosimetry, was mainly adopted.

When the concentration of myosin B was rather high (2.9 mg./ml.), the following interesting phenomena were observed (Fig. 3). By addition of water into or agitation of myosin B solution at the initial stage of recovery (about 5 seconds after the beginning of recovery), the decrease in the intensity of light scattering occurred again, to be followed by gradual recovery towards high level. The addition of arginine (5 mM) or creatine (9 mM) was more effective to the same purpose than

stirring. Addition of lysine (9 mM) or glycocyamine (5 mM) was less effective than arginine. At the later phase, the addition of arginine had considerable effect, but the stirring and the addition of water had little effect upon the optical behavior of myosin B solution. Lysine was less efficient than arginine. Before the addition of ATP or after the complete recovery, even the addition of arginine had no effect on the light scattering of myosin B.

When the concentration of myosin B was so low that the interactions

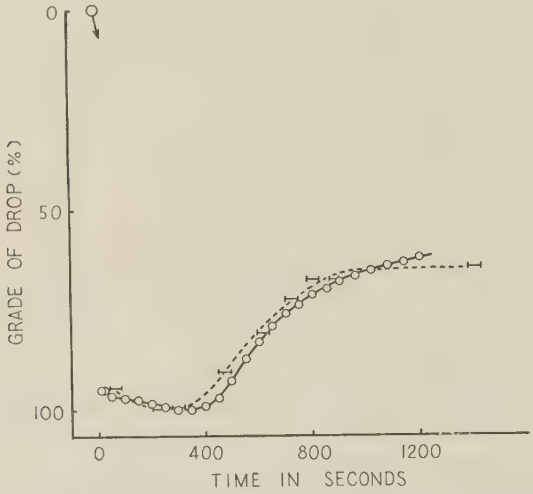


FIG. 2. Change of the viscosity and the light scattering of myosin B solution after the addition of ATP.

♀ indicates addition of 0.4 mM ATP. ○: intensity of light scattering, ⊢: flow time of myosin B solution. 2.7 mg. of protein/ml. 0.6 *M* K^+, 5 mM Mg^{++}. pH 6.7, 13°.

between the protein particles should be negligible (0.19 mg./ml.), even at the early stage of recovery, the addition of arginine, creatine etc., did not exert influence on the recovery process, except the effect of dilution. Therefore it may be concluded that at the initial stage of recovery phase a labile structure is built up by interaction of myosin B particles.

II. ATP Content in the Recovery Process

The change of the quantity of ATP in the recovery phase has already been investigated by several authors, most of whom computed the ATP content from the estimation of P produced by myosin ATPase.

But it is impossible to estimate ATP quantitatively by this method, because almost all ATP added has been hydrolyzed at the recovery phase and, moreover, adenylate kinase is usually contaminated in myosin B preparation. So, in the present experiments the ATP content has been determined after its isolation with ion exchange resin.

Twenty ml. of the reaction mixture which contained 56 mg. of myosin B, 0.6 *M* KCl and 5 mM $MgCl_2$ were injected into a large cubett ($3 \times 3 \times 5$ cm.), placed in the light scattering equipment, and then 0.4 ml. of 5.8 mM ATP was added to the solution. As soon as the light scattering of the reaction mixture which had been kept standing still

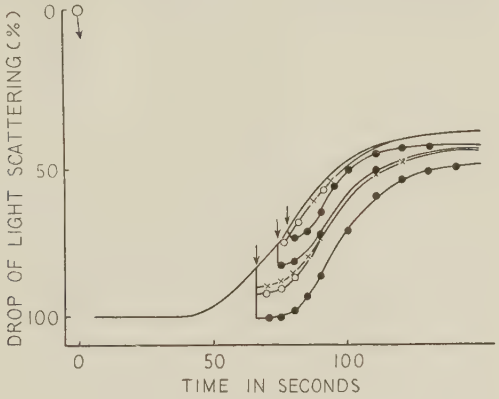


FIG. 3. Influences of various treatments on the early stage of the recovery process.

♀ indicates addition of 6×10^{-5} *M* ATP. ●: addition of 5 mM arginine, ○: addition of H_2O, ×: stirring. 2.9 mg. of protein/ml. Other experimental data as in Fig. 2.

(sample I) or agitated by stirring (sample II) began to rise, the reaction was stopped by the addition of ice cold 20 per cent perchloric acid. This mixture was poured into a test tube in an ice bath with keeping off the decomposition of ATP, and denatured protein was filtered off. The filtrate was neutralized and freed from excess of $KClO_4$ by adding of 1 *N* KOH followed by filtration. Determination of nucleotides in the filtrate was effected by the method of Cohn and Carter.

The results of this experiment are shown in Table I. The most noteworthy point in the results is the fact that the ATP content at the initial stage of the recovery was only 1.8 μM (sample I) or 1.4 μM (sample

II), namely 1/4 or 1/5.4 of the minimum ATP for the maximum drop of the light scattering ($=2.8/4\times10^5\ M=7\ \mu M$, *cf.* (*12*, *18*)). In other words, if ATP remaining at the earliest stage of the recovery is added to myosin B in the presence of Mg^{++}, the degree of the drop (see earlier paper (*12*)) will be only about 20 per cent.

TABLE I

Content of ATP at the Beginning Step of the Recovery Phase
2.8 mg. of protein/ml. 0.6 *M* K^+, 5 mM Mg^{++}, 13°

Sample	Incubation time (*sec.*)	ATP (μM)	ADP (μM)	AMP (μM)
ATP	0	112	7.5	0.4
I	140	1.8	24	93
II	170	1.4	24.5	84

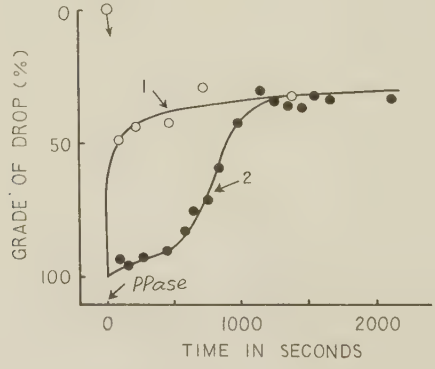


FIG. 4. Effect of PPase on the myosin B-PP system.
⚲ indicates the addition of 1 mM PP. 1: addition of 0.5 unit/ml. PPase, 2: addition of 0.004 unit/ml. of PPase. 0.6 *M* K^+, 2.5 mM Mg^{++}. pH 7.1, 15°.

As contrasted with the results reported in the second paper of this series (See (*12*), Table II), in this case AMP is the main constituent of the reaction products. This may be due to the comparatively higher activity of adenylate kinase of the present sample which is less purified than those used in the previous paper and to the activation of adenylate kinase by the addition of Mg^{++}.

III. Some Observations on the Myosin B-PP System

High KCl Solution—The viscosity of myosin B solution (0.6 *M* KCl) is reduced by the addition of Mg^{++}-PP, but here the viscosity does not recover, because PP is not split by myosin B. When PP was decomposed by the later addition of PPase, the reduction of viscosity was recovered quite easily. The higher the activity of PPase was, the faster the viscosity recovered (Fig. 4).

Low KCl Solution—As has been clearly established, when ATP is added to low KCl solution (0.12 *M*) of myosin B, " superprecipitation " of myosin B is observed (12, 14). However, even when PP (7 mM) added to 0.12 M KCl and 0.4 mM Mg^{++} solution of myosin B was decomposed by the subsequent addition of PPase (0.2 unit/ml.), " superprecipitation " was not observed.

DISCUSSION

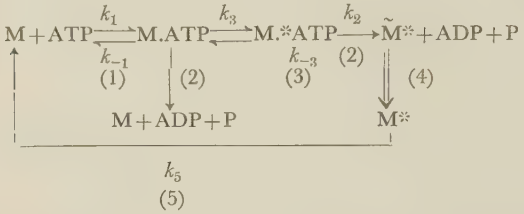
The decline of the light scattering and of the viscosity is attributed to the change of the size and shape of myosin B caused by the combination of ATP with myosin B (*3, 7*). Fast adductor myosin B in the amounts of 4×10^5 g. contains 1 g. mole ATP binding site (*12, 18*), and in the presence of Mg^{++} the combination of ATP with these active sites are so strong that there remains hardly any trace of free ATP, when the content of ATP added is less than that of the active site (*12, 19*).

Hitherto, several investigators have assumed tacitly the following mechanism for the recovery process,

$$\begin{array}{l} M + ATP \rightleftharpoons M.^{*}ATP \\ \qquad\quad \downarrow \leftarrow ATPase \\ \qquad ADP + P \end{array}$$

In this scheme, ATPase active site of myosin B (M) is different from the combining site of ATP necessary to the change of myosin B ($M \rightarrow M^*$), and the recovery occurs simply as a result of decrease, by ATPase, of the concentration of free ATP left in the reaction mixture. However, this view cannot be maintained in the light of the following evidence: (1) If this mechanism were correct, then the content of ATP remaining in the earliest stage of recovery should be almost equal to the minimum ATP content necessary to the maximum drop of the light scattering. As described above, this is not the case. (2) There are some indications that the optical and viscosimetrical changes are the result of the combination of ATP with the ATPase active site (*19*).

From physico- and bio-chemical studies on the myosin B-ATP system, the following reaction mechanism has been postulated by one of the present authors (*3, 19, 20*)[2],



Here, the reaction (1) is the binding of ATP with the ATPase active site of myosin B, the reaction (3) is the change of the size and shape of the myosin B-ATP complex which is accelerated by Mg^{++}. In the absence of EDTA, the activated state of myosin B ($\tilde{M}^*$) is produced by the ATPase-action (2), and, as reported in the previous paper (*11, 12*), arginine and a protein factor are necessary to its change to M* (the step (4)). On the other hand in the presence of EDTA, M*.ATP is changed directly to M* by the ATPase action (*5, 19*). In the usual myosin B, the step (4) is not rate limiting, and the rate of the recovery reaction is determined by the step (5). The steady state law would then give the relation,

$$\frac{[M.^*ATP]}{[M^*]} \sim \frac{k_5}{k_2}.$$

At the earliest stage of recovery phase,

$$[\text{ATP binding site}] \sim [M.^*ATP] + [M^*],$$

and in the presence of Mg^{++},

$$[\Sigma ATP] \sim [M.^*ATP].$$

From these equations,

$$\frac{[\Sigma ATP]}{[\text{ATP binding site}]} \sim \frac{k_5}{k_2+k_5}.$$

As described in the previous section, this ratio was found to be equal to about 1/5. Therefore,

$$k_2/k_5 \sim 4,$$

2) Recently, Nagai (*21*) has proposed a similar mechanism. But his formulation is scanty of experimental evidence and is wanting in critical kinetic examination of the elementary steps.

that is to say, the rate constant of the recovery reaction is much less than that of the ATPase action.[3] This conclusion is supported also by the following observation: the intensity of the light scattering began to rise, in the presence of Mg^{++}, not immediately but after a lag phase of a few seconds, even when the quantity of ATP added was much smaller than that of the ATP binding site.

In the absence of Mg^{++}, on the other hand, the greater part of the ATP remained free ($[\Sigma\ ATP] \sim [ATP]$), because the apparent dissociation constant of the reaction $M+ATP \rightleftarrows M^*.ATP$ is large (*18*, *19*). Accordingly, the relation between the grade of the light scattering change and the content of ATP in the recovery phase becomes to be almost identical with the relation in the drop phase (*cf.* (*12*), Table II and (*18*), Fig. 1).

Recently thixotropic properties of concentrated actomyosin solution were thoroughly investigated by Tonomura and Matsumiya (*23*) and it was found that, in the absence of ATP, a powerful shearing stress was necessary to destroy the structure. In the recovery stage of the myosin B-ATP system a more labile structure seems to be built up, which can be disrupted even by weak agitation. Although the effect of the addition of arginine was much stronger than that due to a stirring, the effect cannot be specific for this substance, because other compounds which have similar structure, *e.g.*, creatine and lysine, could also exert the same influence on the labile state as arginine did.

From the kinetic analysis of the inhibition of myosin B-ATPase and the fall in light scattering induced by PP, it was deduced that these reactions result from the reversible combination of PP with the ATPase

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3) Bailey and Perry (*22*) have suggested that actomyosin (AM) is a stoichiometric complex of myosin (M) and actin (A), that certain centers of M, of which -SH groups form part, possess ATPase activity and that these are also the centers which interate with A. In the presence of ATP, AM is dissociated into A and M;

$$AM \rightleftarrows A+M$$
$$AM+ATP \rightleftarrows A+M.ATP \longrightarrow A+M+ADP+P$$

According to this theory, the results discussed above can be interpreted by postulating that the rate constant of the combination of M with A is about 1/4 of that of the ATPase. But the present authors prefer their theory to Bailey-Perr's one, because in the latter theory there would be some difficulties in accounting for the facts that the viscosity of myosin B solution is not reduced by the elimination of arginine necessary to the recovery process (*12*), and that the relation between the grade of the light scattering drop and the content of ATP added is almost independent on the content of actin (*18*) (*cf.* also (*5*)).

active site of myosin (*3, 10*). On the contrary, Feuer and Wolleman (24) have recently stated, without any experimental basis, that the viscosity drop of actomyosin after the PP addition is an irreversible one. But their supposition cannot be correct, because, as described above, the fall in viscosity caused by PP could be reversed by the later addition of PPase.

As described above, in low salt solution " superprecipitation " could not be observed in the myosin B-PP-PPase system. If the intimate relation of " superprecipitation " of actomyosin with muscle contraction (*14*) be taken into consideration, this fact would seem to lend some support to Weber's theory (*25*), according to which the energy of the energy-rich phosphate bond is transmitted to the contractile system during contraction by the splitting of ATP on the ATPase active site of myosin.

SUMMARY

1. When the concentration of myosin B was rather high, a labile structure was built up at the early stage of the recovery process. This structure could be destroyed by stirring and, more effectively, by the addition of arginine, creatine etc.

2. In the presence of Mg^{++}, the quantity of ATP which remained at the beginning step of the recovery phase was only about 1/5 of the ATP binding site of myosin.

3. The fall of the viscosity of high KCl solution of myosin B after addition of PP was recovered by the subsequent addition of PPase. Even in low salt solution " superprecipitation " could not be observed on the myosin B-PP-PPase system.

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THE INFLUENCE OF INORGANIC IONS ON THE ACTIVITY OF AMYLASES

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It is widely known that the activity of human salivary amylase increases greatly by the presence of Cl^-. This finding has been extended to other animal amylases and also to other inorganic ions such as Br^-, No_3^-, Ca^{++} *etc.* The mechanism of the activation of animal amylases by inorganic anions is a very interesting problem, but at present there are no adequate experimental informations quite enough to explain this mechanism.

If one can completely remove all the ions from the reaction mixture, does the activity of amylase disappear completely? The first report in this field was made by Norris (*1*) using salivary amylase and potato starch dialysed against redistilled water to remove inorganic ions. Under this condition he found that the amylase loses its activity of digesting starch. Recently Bernfeld *et al.* (*2*) studied the influence of inorganic ions on the activities of some crystalline animal amylases, and they found that the crystalline salivary amylase revealed and activity even in the complete absence of inorganic ions which corresponded to 15 per cent of the activity observed in the presence of sufficient amount of NaCl. According to Caldwell (*3*) the dialysed solution of crystalline swine pancreas amylase was activated markedly by the addition of only 10^{-5} *M* NaCl.

In the present study the author used some ion exchange resins instead of dialysis to remove ions more completely. Five kinds of enzymes, namely, human salivary, swine and bovine pancreas, fungal, and bacterial amylases were employed to investigate following points: (1) to what degree the activity of these amylases decreases after deionization; (2) how is the influence of various inorganic salts upon their activity both under the non-ionic condition and in the presence of buffer.

MATERIALS AND METHODS

Amylases from human saliva, swine pancreas, and bovine pancreas were purified

by the procedure of Meyer *et al.* (*4, 5*). *Aspergillus oryzae* (Taka-α-) amylase and bacterial-α-amylase* were recrystallized by the method of Hagiwara (*6, 7*). All the amylase preparations were completely free from maltase activity.

Amylose from potato starch used as substrate was purified by the heat-extractino method of Meyer *et al.* (*8*). It was about 90 per cent purity as examined by the method of Rundle (*9*). For the deionization of the reaction mixture a monobed of 1:3 mixture of Amberlite IR 120 and IRA 400 was employed. The dextrinization activity was measured by observing the change of the " blue value " of iodine reaction which decreases with the progress of the enzyme action. The saccharification was determined by the color reaction of 3,5-dinitrosalicylic acid (*Experiment II*) with the sugar liberated. The details of the procedures will be described later.

RESULTS

Experiment I. Loss of Amylase Activity Caused by Deionization

A salivary amylase solution and a 0.04 per cent amylose solution which came down through column 1 and 2 in Fig. 1 were combined at the junction (A) without exposure to air. This combined liquid was quickly poured with stirring into the flask (B) which contained resin mixture in order to remove a small amount of HCO_3^- and CO_3^{--} absorbed from air. The flask was stoppered immediately, and an aliquot of this liquid was used for the measurement of " blue value."

The grade of deionization of this liquid was tested with the apparatus shown in Fig. 2, by which the electric resistance could be measured before the material was exposed to air. The specific resistance was $1.4\text{–}2.0 \times 10^6\ \Omega$ cm.

The digestion was very slow, because the flask was completely sealed against open air to prevent the absorption of CO_2. If aliquots of the liquid in flask (B) were then taken out and shaken vigorously in the open air, the digestion was explosively accelerated by the formation of a small amount of HCO_3^-, CO_3^{--} from CO_2 in air (Fig. 3).

Thus it was seen that in such a highly deinonized state the reaction was prompted very sensitively by the addition of NaCl. For example, at a concentration of 10^{-2} of NaCl, the activity was enhanced to 2500 times (Table I). Further, the influence of extremely small amount of various inorganic salts was tested and the results are shown in Fig. 4. Even such a low concentration of NaCl as 2×10^{-8} accelerates the reaction velocity vigorously. The greater the concentration of NaCl, the larger the activation effect (Fig. 4). Essentially the same results

* Supplied from Prof. Akabori and Dr. Okazaki.

FIG. 1

FIG. 2

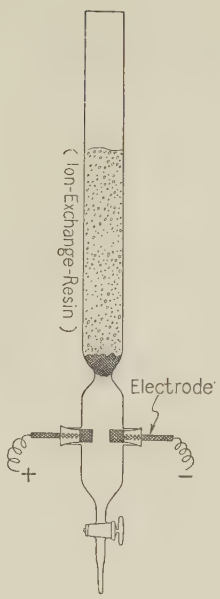

FIG. 3

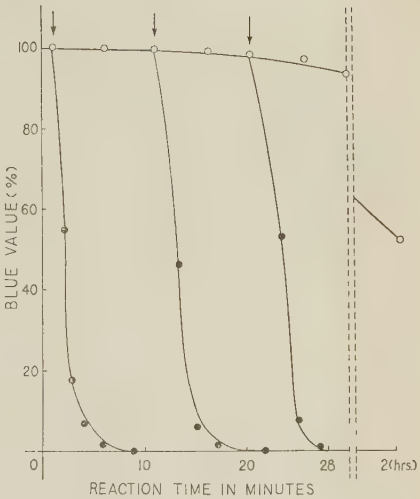

FIG. 1. Apparatus used for deionization of both enzyme and amylose solution by ion-exchange-resins.

FIG. 2. Apparatus used for measurement of conductivity of the deionized specimens.

FIG. 3. Activity of salivary amylase in the non-ionic condition and in the presence of CO_2 in air.

○; in the non-ionic condition. ●; in the influence of CO_2 in air.

The arrows indicate the exposure to air. Experimental conditions; Deionized enzyme 5.0 ml.; deionized amylose (0.04 per cent), 5.0 ml; final volume 10 ml.; At the beginning of the reaction, incubation was carried out in the presence of resine mixture and in a vessel which was completely sealed against air to prevent the absorption of any CO_2 gas in air. Then, aliquots of this liquid in the way of above reaction were taken out at the time which was indicated by the arrows in the figure and shaken vigorously in air to absorb CO_2. And during all this time, at each five minutes, the aliquots of these reaction mixtures were subjected to the extimation of blue value.

as with salivary amylase were obtained with other two amylases.

However, Taka-α-amylase showed quite different properties as shown in Figs. 5 and 6. The influence of the added ions on the enzyme activity was very slight. Bacterial-α-amylase seems to show a character between animal amylases and Taka-α-amylase as shown in Fig. 7. These situations will be later discussed again in more detail.

TABLE I

The Activation Effect of NaCl upon Salivary Amylase

The figures represent the blue values.

Amylase	No. 1			No. 2		
Dilution of amylase solution	×1	×2500	×1	×1	×2500	×2500
Addition of salt	—	10^{-2} *M* NaCl	—	—	10^{-2} *M* NaCl	—
(*mins.*) 0	100%	100	100	100	100	100
5	92	94	—	100	100	—
10	74	77	—	100	100	—
20	30	33	—	98	98	—
60	0	0	—	84	88	—
120	0	0	100	52	45	100

Experimental conditions; Deionized enzyme, 5.0 ml.; deionized amylose (0.04 per cent), 5.0 ml.; final volume, 10 ml.; The incubation was caried out in the presence of resin mixture and in a vessel which was completely sealed against air to prevent the absorption of any CO_2 gas in air. After incubation at 30° 5.0 ml. of $5 \times 10^{-2} N$ NaOH solution was added to each 3.0 ml. of reaction mixture to stop the reaction. Aliquots of this mixture, at the time indicated in the figure, were subjected to the estimation of blue value.

Experiment II. The Effect of Various Inorganic Salts upon the Activities of Amylases in Buffer Solution

To 13 ml. of 0.25 per cent solution of amylose were added 1.0 ml. of the 10^{-1} *M* acetate buffer (pH 5.75) and 1.0 ml. of the salt solution of various concentrations and warmed in a water bath of the constant

temperature at 30°. After 10 minutes 1.0 ml. of the enzyme solution was added quickly. At intervals of one miute from zero time 2.0 ml. of the reaction mixture were taken out and then 5.0 ml. of 5×10^{-2} N NaOH solution were added to stop the digestion. To 3.0 ml. of this

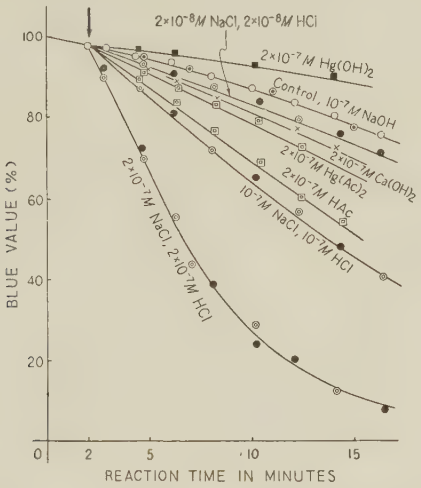

Fig. 4. The influence of inorganic ions upon the activity of salivary amylase in the deionized state.

○, control; $Hg(OH)_2$ solution was prepared from $Hg(CH_2COO)_2$ solution by the treatment with anion exchange resins (Amberlite IRA 400);

Experimental conditions; Deionized enzyme, 5.0 ml.; deionized amylose (0.04 per cent), 5.0 ml.; final volume 10 ml.; At the beginning of the reaction, incubation was carried out in the presence of resine mixture and in a vessel which was completely sealed against air to prevent the absorption of any CO_2 gas in air. Then, aliquots of this liquid in the way of above reaction were taken out at the time which was indicated by the arrows in the figure and added the salt solution and the reactions were continued in air. And during all this time, at each 2 minutes, the aliquots of these reaction mixtures were subjected to the estimation of the blue value.

alkaline mixture 2.0 ml. of 3,5-dinitrosalicylic acid regent (D.N.S.R.) were added according to the procedure of Smith *et al.* From these results obtained the initial velocity of saccharifying reaction and there from the coefficient " α " were calculated as follows in order to express

the influence of inorganic ions upon the activity of amylase;

$$\alpha = \frac{v}{v_0}$$

where v and v_0 represent the initial velocity in the presence and absence of salts respectively. If $\alpha > 1$, it means activation, and if $\alpha < 1$ it means

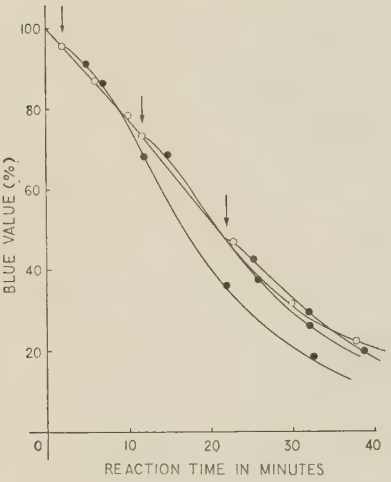

Fig. 5. Activity of Taka-α-amylase in the non-ionic condition and in the presence of CO_2 in air.

○; in the non-ionic condition. ●; in the influence of CO_2 in air.

The arrows indicate the exposure to air. Experimental conditions; the same as Fig. 3.

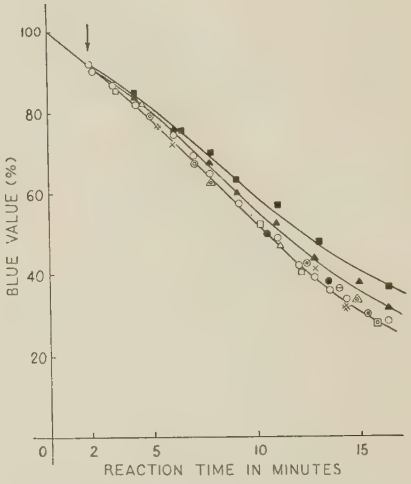

Fig. 6. The influence of inorganic ions upon the activity of Taka-α-amylase in the deionized state.

○ control ◉ $Hg(OH)_2$ △ $Cu(OH)_2$
● HNO_3 # CH_3COOH □ H_2SO_4
▲ Na_2SO_4 ◬ HCl × $NaNO_3$
◉ CH_3COONa

The arrow indicates the addition of salts. The concentration of salts; 2×10^{-7} *M*. Experimental conditions; the same as Fig. 4.

inhibition, while $\alpha = 1$ means no influence. In the colorimetric estimation of the sugar the color development was found to be much interfered with the presence of some ions such as Mn^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Ca^{++}, Sn^{++}, Sn^{++++} *etc.* Therefore these ions were removed from the reaction mixture by the following ways before applying the D.N.S.R.

Ca^{++} ..... removed by oxalate
CN^- ..... aerated off from acidified solution
NO_2^- ..... $NH_2HSO_3+HNO_2=H_2O+N_2\uparrow+H_2SO_4$
SCN^-..... $3SCN^-+10H^++13NO_3^-=16NO\uparrow+3CO_2\uparrow+3SO_4^-+5H_2O$
Mn^+, Fe^{++}, Fe^{+++}, Co^- etc. ..... removed by H_2S gas.*

The pH value of the reaction-mixture was kept at 5.75, because the measured optimum pH of the five kinds of amylases were around 5.75.

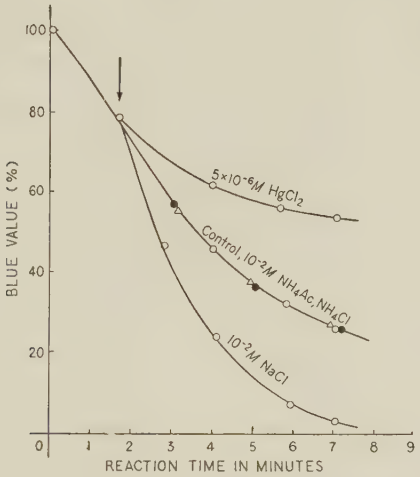

FIG. 7. The influence of inorganic ions upon the activity of bacterial-α-amylase in the deionized state.

○ control; The arrow indicates the addition of salts. Experimental conditions; the same as Fig. 4.

The results of *Experiment II* are shown in Table II. Generally the animal amylases were sensitive to inorganic ions, while Taka-α-amylase was not the case, and no activator was found acting upon the latter. In respect of the ion sensitivity the bacterial-α-amylase was situated between the animal and Taka-α-amylase. Hg^+ was a strong inhibitor common to the five kinds of amylase.

* The colloidal solution of amylose has a strong power to stabilize and disperse the metal sulfides suspension and colloidal sulfur formed. But it was found that Rochelle salt is very effective to precipitate these colloidal particles.

Table II

The Influences of Inorganic Salts upon the Activity of Amylases in the Presence of Acetate Buffer

$$pKi = -\log_1 \frac{\alpha}{1-\alpha} \times [I]$$

$\alpha = v/v_0$, where v and v_0 represent the initial velocity in the presence and in the absence of salts, respectively. Experimental conditions; Deionized enzyme, 1.0 ml.; 0.25 per cent amylose, 13 ml.; 2×10^{-1} *M* acetate buffer (pH 5.75), 1.0 ml.; salt solution, 1.0 ml.; final volume, 16 ml.; After incubation at 30°, 5.0 ml. of 5×10^{-2} *N* NaOH solution was added to each 3.0 ml. of the reaction mixture to stop the reactions. Aliquots of this mixture were subjected to the estimation of sugar liberated, by which the initial velocities of the reactions were calculated.

Human salivary amylase

Salt	Concentration	$\alpha = v/v_0$	*pKi*
$CaCl_2$	10^{-2} *M*	11.70	—
NaCl	" "	9.74	—
$Ca(CH_3COO)_2$	" "	1.50	—
Na_2SO_4	" "	1.03	—
$(NH_4)_2SO_4$	" "	1.00	—
$Na_2C_2O_4$	" "	0.48	2.03
$Hg(CH_3COO)_2$	4×10^{-6} "	0.67	5.10

Human salivary amylase

Activation	Cation	$Ca^{++} > Sr^{++} > Mg^{++} > Co^{++} > Ni^{++} > Ba^{++} > Na^+ > K^+ \fallingdotseq Rb^+ \fallingdotseq Cs^+ \fallingdotseq Li^+$
	Anion	$Cl^- > Br^- > NO_2^- > I^- > F^- > H_2PO_4^{--}$
Inhibition	Cation	$Hg^{++} > UO_2^{++} > Cu^{++} > Ag^+ > Fe^{+++} > Pb^{++} > Fe^{++} > Cd^{++} > Zn^{++} > Al^{+++} > Cr^{+++} > La^{+++}$
	Anion	$C_2O_4^{--} > SCN^- > P_2O_7^{--}$
Inactive		CH_3COO^-, HCO_3^-, CN^-, SO_4^{--}, NH_4^+

Bovine pancreas amylase			
Salt	Concentration	$\alpha = v/v_0$	pKi
$CaCl_2$	10^{-2} M	11.03	—
NaCl	„ „	9.00	—
$Ca(CH_3COO)_2$	„ „	1.43	—
Na_2SO_4	„ „	1.01	—
$(NH_4)_2SO_4$	„ „	1.01	—
$Na_2C_2O_4$	„ „	0.58	1.86
$Hg(CH_3COO)_2$	4×10^{-6} „	0.69	5.00

Swine pancreas amylase			
Salt	Concentration	$\alpha = v/v_0$	pKi
$CaCl_2$	10^{-2} M	10.80	—
NaCl	„ „	8.70	—
$Ca(CH_3COO)_2$	„ „	1.50	—
Na_2SO_4	„ „	1.03	—
$(NH_4)_2SO_4$	„ „	1.00	—
$Na_2C_2O_4$	„ „	0.87	1.17
$Hg(CH_3COO)_2$	2×10^{-5} „	0.48	4.74

Taka-α-amylase			
Salt	Concentration	$\alpha = v/v_0$	pKi
$HgCl_2$	1.33×10^{-5}	0.67	4.88

Taka-α-amylase

Activation	None	
Inhibition	Cation	$Hg^{++}>Ag^{+}>Pb^{+}>Fe^{+++}>Sn^{++}>Sn^{++++}>Al^{+++}>Cd^{++}>Zn^{++}>Mn^{++}>Co^{++}>Ni^{++}>Ca^{++}>Fe^{++}>La^{+++}$
	Anion	None
Inactive	Li^{+}, Na^{+}, K^{+}, Rb^{+}, Co^{+}, Be^{++}, Mg^{++}, NH_4^{+}, Cl^{-}, Br^{-}, I^{-}, F^{-}, SO_4^{--}, Ac^{-}, NO_3^{-}, HPO_4^{--}, $C_2O_4^{--}$, CN^{-}, SCN^{-}	

Bacterial-α-amylase

Salt	Concentration	$\alpha = v/v_0$	pKi
NaCl	10^{-2} *M*	1.14	—
$(NH_4)_2SO_4$	" "	1.01	—
$Na_2C_2O_4$	" "	0.99	—
$Hg(CH_3COO)_2$	10^{-5} "	0.87	4.17

Bacterial-α-amylase

Activation	Cation	$Na^{+}>Li^{+}\fallingdotseq Rb^{+}\fallingdotseq Cs^{+}\fallingdotseq K^{+}$
	Anion	Cl^{-}
Inhibition	Cation	$Hg^{++}>Cu^{++}>Ag^{+}>Fe^{+++}>Pb^{++}>Sn^{++}>Zn^{++}>Sn^{++++}$ $Fe^{++}>Al^{+++}>Cd^{++}>La^{+++}$
	Anion	None
Inactive	Be^{++}, Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Cr^{+++}, Mn^{++}, Co^{++}, Ni^{++}, NH_4^{+}, F^{-}, Br^{-}, I^{-}, SO_4^{--}, NO_3^{-}, HPO_4^{--}, $C_2O_4^{--}$, CN^{-}, SCN^{-}	

Experiment III. The Influence of Inorganic Ions upon Optimum pH of Salivary Amylase

Procedure of this experiment was almost identical with that of *Experiment II* except using the mixture of acetate and phosphate buffer of pH between pH 4.0 and 9.5 instead of acetate buffer alone. The results are shown in Fig. 8. The optimum pH of deionized salivary amylase was 5.75. By adding of 10^{-2} *M* NaCl, NaBr, the optimum pH

shifted to 6.80, and by adding of NaI and $NaNO_3$, it shifted to 6.3 and 6.50 respectively. In case of cations such as Ca^{++}, Hg^{++} *etc.*, the shift of optimum pH was not observed, but only the heights of the curves were changed. Similar results were obtained in the other two animal amylases.

Experiment IV. The Influence of Inorganic Ions upon the Dextrinizing and Saccharifying Action of Amylases

To 13 ml. of 0.25 per cent amylose were added 1.0 ml. of 2×10^{-2} *M* acetate buffer and 1.0 ml. of salt solution, and the reaction was started

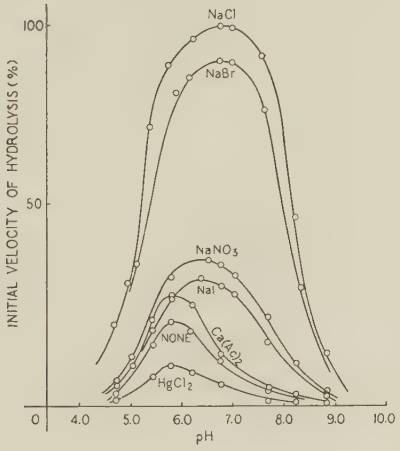

FIG. 8. The influences of inorganic ions upon optimum pH of salivary amylase. The initial velocity of the hydrolysis with the addition of NaCl at pH 6.8 taken as 100. Experimental conditions; The mixture of $2 \times 10^{-1}M$ acetate- and phosphate-buffer was used. Other conditions are the same as Table III.

by adding 1.0 ml. of deionized enzyme solution at 30° as described in *Experiment II.* To the aliquot of 2.0 ml. taken out from the reaction mixture were added 5.0 ml. of 5×10^{-2} *N* NaOH and 3.0 ml. of this alkaline mixture was used for the color-development by D.N.S.R., while 0.5 ml. was used for determining the " blue value " at 610 mμ. Thus the dextrinization-saccharification-curve (*12*) was constructed by plotting the value of dextrinization on abscissa and the value of saccharification on ordinate (Fig. 9). The shape of this curve was not influenced at all by pH and enzyme concentration. Now, if the influence of

inorganic salts upon each of the two actions of amylase was different, the curve would be expected to shift upward or downward. However, the experimental results in Fig. 9 showed no such shift of the curve by the addition of the inorganic ions.

Experiment IV. The Interactions among Various Inorganic Ions

In this section the simultaneous action of two or more kinds of ions upon the amylase activities were studied. The procedure was almost

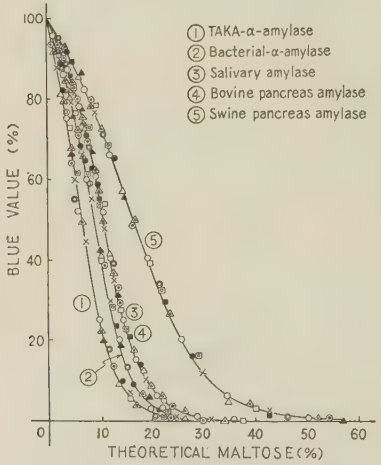

Fig. 9. The dextrinization-saccharification-curves of amylases and the influences of various inorganic salts upon them.

○ control (pH 5.75) △ pH 5.00 ⊙ enzyme concentration $\frac{1}{8}$ • $HgCl_2$ (2×10^{-5} *M*), □ Co $(CH_3COO)_2$ (10^{-2} *M*), ⊡ Na_2SO_4 (10^{-2} *M*), ⧋ $AgNO_3$ (5×10^{-5} *M*), ▩ $Fe_2(SO_4)_3$ (5×10^{-4} *M*), × $CuSO_4$ (5×10^{-5} *M*), ▲ $MnSO_4$ (10^{-2}*M*), ⧋ NaCl (10^{-2}*M*), ⊡ $CaCl_2$ (10^{-2}*M*)

(all of these are final concentrations)

Experimental conditions; the same as Table III. Sugar liberated and blue value were estimated.

identical with that of *Experiment II.* The results are shown in Table III. A clear competition between cations and anions was recognized. These data will be discussed later.

In this experiment, the influences of buffer ions were also observed.

DISCUSSION

The activity of Taka-α-amylase remained almost unchanged even

Table III

The Effects of Various Inorganic Ions upon Amylase of Human Saliva, Swine Pancreas and Bovine Pancreas

10^{-2} *M* $Ca(CH_3COO)_2$	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
" $CaCl_2$	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
" NaCl	−	−	+	−	+	+	−	+	−	+	−	−	−	−	+	−	−	+	−	−	−
" NaBr	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" $Ba(CH_3COO)_2$	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−
" $BaCl_2$	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
10^{-5} *M* $Hg(CH_3COO)_2$	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	−	+
10^{-2} *M* $Na_2C_2O_4$	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
" $ZnSO_4$	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
" Acetate-buffer	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
α	5×10^{-3}	1.00	9.74	7.80	11.20	11.20	1.51	11.00	11.50	12.00	2.5×10^{-3}	0.67	0.84	0.52	9.10	1.38	7.81	8.20	0.50	0.41

* 10^{-7} *M*

(+) represents the addition of the respective salt, and (−) represents no addition of it.

$\alpha = v/v_0$, where v and v_0 represent the initial velocity in the presence and in the absence of salts, respectively. Experimental conditions;

Deionized enzyme, 1.0 ml.; 0.25 per cent amylose, 13 ml.; 2×10^{-1} *M* acetate buffer (pH 5.75) or deionized water, 1.0 ml.; salt solution, 1.0 ml.; final volume, 16 ml.; After incubation at 30°, 5.0 ml. of 5×10^{-2} *N* NaOH solution was added to each 3.0 ml. of the reaction mixture to stop the reactions. Aliquots of this mixture were subjected to the estimation of sugar liberated, by which the initial velocities of the reactions were calculated.

after extensive deionization. This is a characteristic property of Taka-α-amylase as contrasted to that of salivary and pancreas amylases.

The activity of salivary amylase which had been inactivated by deionization was extensively restored by adding very small amount of NaCl, and even by atmospheric CO_2 markedly. Various inorganic ions tested were not effective upon both deionized and un-deionized Taka-α-amylase. The sensitivity of the bacterial α-amylase towards inorganic ions was higher than that of Taka-α-amylase but lower than that of salivary or pancreas amylase (*13*).

Fig. 4 indicates that Cl^- activates the salivary amylase independently from the presence of Na^+. Similary, the activation by $Hg(CH_3COO)_2$ at a concentrations of 10^{-7} *M* might be the result of the summation of inhibitory effect of Hg^{++} and a predominant activating effect of CH_3COO^-. The inhibition at higher concentrations such as 10^{-5} *M* or more may be caused by the overcoming of inhibitory effect of Hg^{++} over the activating effect of CH_3COO^-. The activation of salivary amylase by cations such as Ca^{++} *etc.* seems to be of different nature from that of anions.

Myrbäck (*14*) proposed a hypothesis as to the mechanism of activation of salivary amylase by anions such as Cl^-, Br^-, No_3^-. According to him, the amino groups in the protein molecule are essential to the enzyme action, and some amount of these groups would be unmasked in the presence of Cl^- *etc.* This assumption is based on the fact that the optimum pH of salivary amylase shifts to alkaline side when the anions are added to the reaction mixture.

In the present study the optimum pH of the salivary amylase was found to be unchanged by the addition of Ca^{++} *etc.* The author considers that the mechanism of activation by cations and anions may be different. This consideration was further confirmed by the interaction among various inorganic ions. Table III represents the results of the interacting effect of (1) both cations and anions, (2) cations, and (3) anions. The results show clearly that the competitive interaction occurs only between cations and anions. The activating action of anions drive away not only the inhibiting action of cations such as Hg^{++}, Zn^{++}, but also the activating action of cations such as Ca^{++}, Ba^{++}. These findings also suggest that the mechanisms of action may be quite different between cations and anions.

Table III shows that the activity in the presence of 10^{-2} *M* acetate buffer alone is 200 times larger than that without any addition of ions, and that the effects of salts on the values of "α" became very slight

in the presence of acetate buffer. Table III shows also that the *pKi* of Hg^{++} drops from 7.0 to 5.10 in the presence of buffer.

These facts would be also explained by the above assumption.

Akabori (in 1955) reported that EDTA (Ethylene-Diamine-Tetra-Acetate) remove Ca from the crystalline Taka-α-amylase. He suggested that the ion exchange resins might attack the enzyme molecules by the similar mechanism causing their denaturation or decomposition. According to the present experiment, it would be considered that the effect of deionization by ion exchange resins may be identical to that of dialysis against destilled water and does not cause their denaturation.

Experiment IV indicates that the inorganic ions tested exert influence upon both dextrinizing and saccharifying activity to the same extent. From this point of view it might be said that the two types of the reaction (dextrinizing and saccharifying) is caused by a single action of the enzyme.

Ono *et al.* (*15*) reported that Ca^{++} affected preferentially the saccharifying action of bacterial-α-amylase and so the dextrinization-saccharification-curve shifted downward in proportion to the concentration of Ca^{++}. However, it must be noted that the color development by D.N.S.R. is much depressed by the presence of Ca^{++} as indicated in Experiment II. After the correction of this depressive effect of Ca^{++}, the author found that Ca^{++} promoted both actions of the amylase at the same rate.

SUMMARY

1. The effect of strong deionization from the amylase]solutions upon their activities is summarized as follows ;

(a) The activity of human salivary amylase deionized by ion exchange resing was only 0.04 per cent of the activity displayed in the presence of a sufficient amount of NaCl, and the decreased activity was recovered even by an extremely small amount of various ions, for example, 10^{-8} *M* of NaCl.

(b) The activity of Taka-α-amylase was not influenced at all by treatment with ion exchange resin.

(c) Bacterial-α-amylase showed a behavior toward inorganic ions which situated between animal amylases and Taka-α-amylase.

2. Ions in salt solution act completely independently. Thus the activation by NaCl of salivary amylase is due solely to Cl^-, and not to Na^+ at least when the concentration of NaCl is low.

3. The action of about 40 kinds of inorganic salts upon activities of the amylases was studied in the presence of buffer ions.

4. It was shown that both the "dextrinizing action" and the "saccharifying action" of amylases were influenced by various inorganic salts to an equal extent.

The author wishes sincerely to dedicate many thanks to Prof. Shimazono of this department for his valuable instruction during this experiment and Miss Watanabe, the Department of Biochemistry, Institute of Public Health, for her technical assistance. The author wishes to express his thanks to Prof. Akabori and Dr. Okazaki for their kind supply of the samples.

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ON THE SPECIES DIFFERENCE IN CARBOXY-TERMINAL AMINO ACIDS OF SERUM ALBUMINS FROM VARIOUS ANIMALS

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The relation between species specificity and chemical structure of proteins having the common biochemical functions is a very interesting problem. In order to elucidate this problem, Ozawa and Satake (*1*) studied on the amino acids composition of the N-terminal and its vicinity of the hemoglobins from ten kinds of animals. They found that most of the samples had the same N-terminal amino acid and the same amino acids sequence in the vicinity of the N-terminal of the proteins but a few exceptions. However, none of the studies has been made concerning the relation between species specificity and C-terminal amino acids.

In this paper, the analysis of C-terminal amino acids of serum albumins from eight kinds of mammalians; human, dog, rabbit, horse, pig, bovine, sheep and goat, was performed by the hydrazine method of Akabori *et al.* (*2-5*). The results thus obtained showed that among the eight different samples, five had alanine as the C-terminal amino acid and the other had leucine instead. Incidentally the animals having alanine as C-terminal in serum albumin belong to the same zoological classification, *Ungulata*.

MATERIALS

Anhydrous Hydrazine—50 g. of 80 per cent hydrazine hydrate, 500 g. of toluene and 500 g. of calcium oxide were mixed in a glass vessel and stood over night putting a joint stopper on. The content was then refluxed for three hours with a sodium hydroxide tube fixed at the top of a reflux condencer. Hydrazine thus anhydrated was distilled as the azeotropic mixture with toluene at 93–94°. The lower layer of distillate could be used as anhydrous hydrazine without any further purification for the hydrazinolysis of protein.

Enantaldehyde—Raw enantaldehyde was prepared by thermal decomposition of

castor oil in the presence of a small amount of talc. It was then purified with the usual techniques of aldehyde purification, *i.e.*, distillation, precipitation with sodium bisulfite, decomposition of the addition compound and redistillation under diminished pressure. The yield was 5 per cent.

Serum Albumins—Serum albumins were prepared by the addition of saturated ammonium sulfate solution to the sera from the various animals. The crystals thus obtained were recrystallized two or three times. They were dialysed against distilled water in order to remove ammonium sulfate and lyophilized. The preparations were stored and used for the assay.

EXPERIMENTAL

General Procedure—The procedure used in this study was similar to that employed by Ohno (*3, 5*) except the use of enantaldehyde instead of isovaleraldehyde. A brief description of the general procedure is as follows.

70 mg. of serum albumin (*ca.* 1 μ mole) was added to 1.5 ml. of anhydrous hydrazine in a dry glass tube. The tube was sealed and heated in a boiling water bath for ten hours usually. After the reaction, the mixture was evaporated in a vacuum desiccator containing concentrated sulfuric acid. The residue was washed with every 1 ml. of water three times. The aqueous solutions combined were put into a separation funnel together with 0.5 ml. of the enantaldehyde and shaked for 30 minutes. The upper enantaldehyde layer containing the complex of the amino acid hydrazides and enantaldehyde was separated off. The same process was repeated with the aqueous part once more, using 0.5 ml. of the aldehyde. When the aqueous solution was turbid after the second extraction, centrifugation is recommeded to clarify. The aqueous solution was then twice washed with ethyl acetate, 3 ml. at each time to eliminate the enantaldehyde. The dinitirophenylation was followed by the addition of 0.3 g. of sodium bicarbonate and 6 ml. of 2 per cent DNFB. After shaking for 90 minutes, the reaction mixture was diluted with ten times volume of water, acidified with 2 *N* hydrochloric acid, and extracted with ethyl acetate three times using 30, 20, and 20 ml. at each time. The ethyl acetate solution combined was extracted three times with 2 per cent sodium bicarbonate solution (20, 10 and 10 ml.). The aqueous solution was again acidified and extracted three times with ether (10, 5 and 5 ml.). The etheral extracts were combined, and then evaporated *in vacuo*. From the residue, dinitrophenol was sublimated on a cold finger under the condition of 80° and 1 mm. pressure (*6*). In order to remove dinitrophenol perfectly, the residue was again dissolved in ether or acetone, followed the evaporation and the sublimation in the same way as described above. This process was repeated three times. The final etheral or acetone solution containing the residue was transferred to filter paper (Toyo filter paper No. 51) and developed in accordance with the two-dimentional chromatography. The primary solvent was prepared by fractionating the upper layer from the mixture consisting of 1.4 per cent ammonia solution: butanol (1:1) and the secondary solvent was 1.5 *M* phosphate buffer of pH 5.6 (*7*). Identification of DNP-derivatives of amino acids

was carried out by comparing their R_f values to those of the known amino acid derivatives under the same condition. To make the identification more plausible the yellow spot was eluted and the chromatography of the solution was carried out again, sometimes in the presence of the DNP-derivatives of the amino acids in question.

Quantitative Estimation—Part of the paper where DNP-amino acid was adsorbed was cut off, eluted with water and the amount of the corresponding amino acid was calculated from the optical density at 360 mμ. In order to make a minor correction due to the filter paper used, the optical density measurement of the aqueous eluate from nearly the same area of the same filter paper used for the chromatography was carried out. The recovery of the DNP-amino acid corresponding to C-terminal was, however, neither large enough nor reproducible in this method. The direct estimation of the C-terminal amino acid by this method therefore seemed to be unsatisfactory. When the hydrazinolysis was carried out for ten hours with DNP-Leu and DNP-Ala, the recoveries were not good and fluctuated considerably. With many experiments, however, using the mixture of DNP-Ala, the DNP-Leu, the ratio of DNP-Ala and DNP-Leu thus obtained remained nearly constant (See Table I).

TABLE I

The Recovery of DNP-Ala and DNP-Leu, and the Molar Ratio of DNP-Ala to DNP-Leu

DNP-Ala	40—60%
DNP-Leu	45—65%
DNP-Ala/DNP-Leu	0.87—0.91

The time of hydrazinolysis was ten hours.

The quantitative estimation of DNP-amino acid, therefore, was carried out in the following way. As it will be shown later, the C-terminal amino acids were found either alanine or leucine. When the C-terminal amino acid of a protein was considered alanine, a certain amount of the standard leucine solution was added as a tracer, while on the other hand, when the C-terminal was observed leucine, alanine was added. In these cases, the molar ratio of the albumin (molecular weight was assumed 68,000) to the added standard amino acid was adjusted to be approximately unity. First a certain amount of the added standard amino acid solution was evaporated to dryness in a glass tube and then the corresponding amount of protein and anhydrous hydrazine were added and the tube was sealed. The same procedure including hydrazinolysis, dinitrophenylation and colorimetory of DNP-amino acids *etc.*, was carried out as described above. In order to estimate equivalents of the terminal amino acid, the molecular ratio of the DNP-amino acids due to the protein terminal to the DNP-derivative of the added standard amino acid was measured, after taking account of a small correction (*ca.* 10 per cent) due to the yield difference between DNP-derivatives of alanine and leucine.

Preparation of Anhydrous Hydrazine by Toluene Method—There is a danger of explosion with the usual preparation of anhydrous hydrazine using distillation in a copper flask (*8*). The yield by this method is about 65 per cent. On the other hand, the toluene method which is described in this paper seems to be superior because there is no danger of explosion during the purification process which can be done in glass joint vessels. The yield of anhydrous hydrazine was 75 per cent when the fresh toluene was used at the first time while the yield increased up to 95 per cent from the second time using the toluene which was obtained from the lower layer of the distillate at the first distillation and can be stored in a sodium hydroxide desiccator for repeated use. Purity of anhydrous hydrazine thus obtained was 98.5 per cent and no trouble was encountered at the hydrazinolysis of the proteins.

Characteristics of Aldehydes—Hitherto, salicylaldehyde, isovaleraldehyde or benzaldehyde have been used. Under the experimental conditions, however, part of the amino acid hydrazides derived from these aldehydes appeared in crystalline state, thus making the separation of the hydrazides rather troublesome. In order to avoid this difficulty enantaldehyde was employed instead. In this case, no crystallization was observed and the upper aldehyde layer containing the complex of enantaldehyde with amino acids hydrazides was fractionated quite easily from the lower aqueous layer which contained C-terminal amino acids. Furthermore, enantaldehyde did not form peroxide during its storge like benzaldehyde. Due to these characters, enantaldehyde was shown to be more excellent than salicylaldehyde at the hydrazinolysis of lysozyme. When salicylaldehyde was used after the hydrazinolysis, the yield of leucine as C-terminal was only 52 per cent of the yield when enantaldehyde was used.

RESULTS AND DISCUSSION

As shown in Table II, the C-terminal amino acids observed for the serum albumins from various mammalians were either leucine or alanine. It is very interesting to see that animals having alanine as C-terminal of serum albumin belong to a special zoological class called *Ungulata*.

The results obtained for human and bovine serum albumins did not contradict with the experimental result using carboxypeptidase (*9*) to liberate C-terminal amino acids as well as with the result of hydrazinolysis of bovine serum albumin by Niu and Fraenkel-Conrat (*10*). Studies showing the exchange of amino acid components among the classifically closer species have been reported. The amino acid sequences of vasopressin from bovine and pig, belonging to *Artiodactyla*, are similar except the second amino acid from the C-terminal (*11*) which is common for both species, glycine amide.

bovine vasopressin	-Pro-*Arg*-Gly NH_2
pig vasopressin	-Pro-Lys-Gly NH_2

The amino acid components next to the common N-terminal amino acid of hemoglobin from guinea pig and rabbit which belong to *Todentia* (*1*) are exchanged as follows:

guinea pig hemoglobin	Val-*Ser*-
rabbit hemoglobin	Val-Gly-

The results of splendid work (*12*) concerning the amino acid sequences of insulins from sheep, bovine and pig belonging to *Artiodactyla* have shown that the 8th to 10th components from the common N-terminal amino acids, glycine, are exchanged as follows

sheep insulin	-Ala-*Gly*-Val-
bovine insulin	-Ala-Ser-Val-
pig insulin	-*Thr*-Ser-*Ileu*-

TABLE II

C-Terminal Amino Acids of Serum Albumins from Mammalians

Classification		C-terminal amino acids
Orders	Species	
Primalter	Human	Leu (1)
Carnivora	Dog	Leu *
Todentia	Rabbit	Leu *
Ungulata		
(*Perissodactyla*)	Horse	Ala (1)
(*Artiodactyla*)	Bovine	Ala (1)
	Goat	Ala (1)
	Sheep	Ala (1)
	Pig	Ala (1)

The figures in the brackets are the number of C-terminal amino acids per unit molecule of protein. * The quantitative analysis could not be undertaken.

The similar specific difference in amino acid sequence has been shown among suborders or orders higher zoological classification as well as among species. For example among the N-terminal amino acids of hemoglobin from the animals (bovine, sheep and goat) belonging to *Ruminantia* are found methionine, whereas those from the other only show valine as the N-therminal. (*1*, *13*).

The result reported here is to add one more example to the problem concerning the specific difference in chemical composition of proteins.

serum albumin from *Ungulata*	-*Ala*
serum albumin from the others	-Leu

The author wanted to decide that this difference was due to whether the exchange of the amino acids, alanine and leucine, or the lacking of alanine or leucine, namely,

Ungulata	-Ala (lacking of leucine)
the others	-Ala-Leu

In order to decide this question we must know the amino acids next to the C-terminal, but the second amino acids from C-terminal was not identified by means of the partial hydrazinolysis 100°, 1 hour or 65°, 3 hours.

The exchange of alanine and leucine is found in the corticotropin from sheep and pig (*14*). The 9th amino acids from C-terminal of sheep and pig corticotropin are alanine and leucine, respectively. So the difference recognized at C-terminal amino acids of serum albumins may be the similar exchange.

Serum albumins from *Ungulata* have the same C-terminal amino acid, alanine, but the second amino acids from N-terminal are different with bovine and horse (*15*, *16*), that is

bovine serum albumin	Asp-*Thr*- ..... -Ala
horse serum albumin	Asp-Ala- ..... -Ala
human serum albumin	Asp-Ala- ..... -Leu

The specificity may be formed by the difference of amino acids among the species and/or orders, as described above.

It is very interesting to consider such a species, or order specificity in amino acids composition in connection to the classification and evolution of animals.

SUMMARY

1. A simple preparation method of anhydrous hydrazine using toluene was described.

2. Enantaldehyde was favorably employed to remove the hydrazides of amino acids.

3. The C-terminal amino acids of serum albumins were found leucine, with various mammalians, except the *Ungulata* where alanine was found as the C-terminal.

The author wishes to express his thanks to Professors S. Akabori and K. Satake for encouragement throughout the course of research and to the Teikoku Hormone Manufacturing Co. Ltd. and to the National Institute of Agricultural Sciences, Chiba, for the supply of the bloods used for the preparation of albumins.

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MICROBIOLOGICAL DEGRADATION OF BILE ACIDS
XI. ON THE DECOMPOSITION OF CHOLIC ACID BY A SOIL BACTERIUM

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In a previous paper (*1*) of this series, it has been demonstrated that a Gram-positive soil bacterium (CE-1), cultured in a synthetic medium containing cholic acid as the sole source of carbon, oxidized cholic acid to 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid.

Recently a Gram-negative bacterium which was also able to grow on the same medium was isolated from the soil of a slaughter-yard. The present report is designed to yield informations (a) on the isolation of the cholic acid-oxidizing organism from soil and (b) on the degradation product of cholic acid by this organism.

EXPERIMENTAL

Isolation and Characteristics of Cholic Acid-Oxidizing Organism—A synthetic culture medium which was described in the first paper (*2*) of this series was adopted in this experiment. Several pure strains of bacteria which were able to grow in the medium were obtained by the same manner as described in a previous paper (*1*).

One of the strains (CF-1) used in this experiment was a Gram-negative and nonsporulating rod which grew well at 37° in the range of pH 6.5 to 7.5.

Full details of the identification and further characterization of this organism will be reported later.

Cultivation and Isolation of Degradation Products—Twenty-seven 500 ml. bottles, each containing 250 ml. of the above-described synthetic medium which dissolved 0.1 g. of cholic acid per 100 ml., were autoclaved at 110° for 20 minutes and inoculated with the strain CF-1. The inoculated bottles were incubated stationarily at 37° until Pettenkofer's test of the culture supernatant gave a negative result.

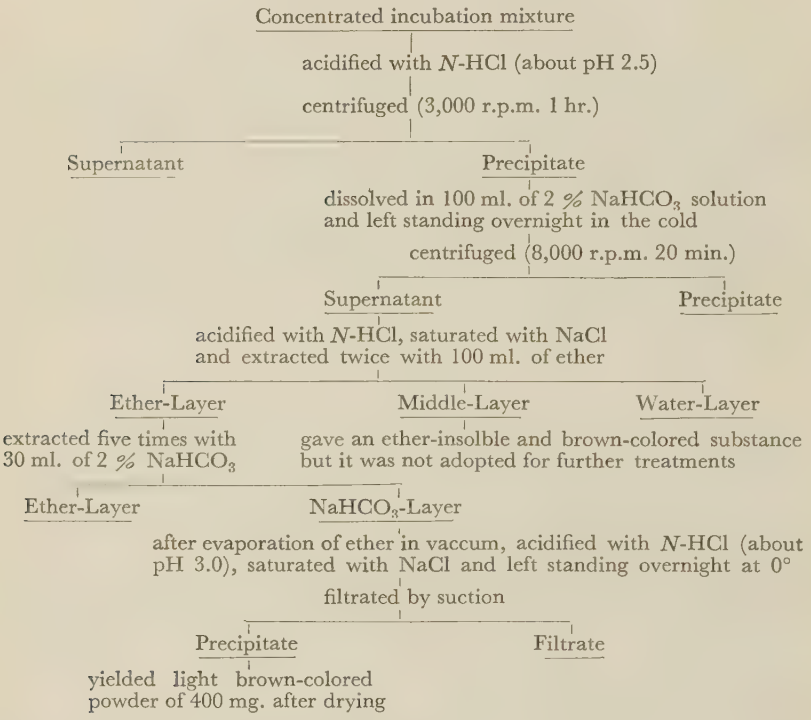
Incubation for 22 or 27 days was necessary to give the negative

Pettenkofer's test. At the end of incubation period the pH of the culture dropped to about 6.0 from original pH 7.2.

The combined supernatant was 6,520 ml. of a pale yellow viscous solution. The pH of the solution was adjusted to about 6.8 with dilute sodium bicarbonate solution and the neutralized solution was concentrated to about 250 ml. in vacuum below 50°. The concentrated so-

DIAGRAM 1.

Isolation of the Degradation Products Obtained after Incubation of Cholic Acid with the Strain CF-1



lution was treated as shown in Diagram 1.

The final brown precipitate described in Diagram 1 was recrystallized from methanol—ethyl acetate and 200 mg. of pale brown-colored rods with m.p. 178–182° was obtained.

Because of the unsuccess in further purification, the methyl ester of this acid was prepared as follows.

Methyl 7α,12α-*Dihydroxy*-3-*keto*-Δ^4-*cholenate*—The methyl ester of the above acid with m.p. 178–182° was prepared with diazomethane by the usual manner and crystallized from ethyl acetate, in plates, m.p. 178–179°, $\lambda_{max.}^{alc.}$ 243 mμ (log ε 3.95). This ester gave no depression with a Eguchi's sample (*1*) with m.p. 178–179°. The infrared spectrum of this ester showed the following absorption bands (in Nujol): hydroxyl, 2.91 and 3.00 μ; ester, 5.74 μ; Δ^4-3-ketone, 6.07 and 6.27 μ.

Analysis Calcd. for $C_{25}H_{38}O_5$: C, 71.74; H, 9.15.
Found: C, 71.27; H, 9.20.

Methyl 12α-*Hydroxy*-3-*keto*-$\Delta^{4,6}$-*choladienate*—In the same manner as described in a previous paper (*1*), the above ester was converted into the known dienone ester as follows: a solution of 28 mg. of the above ester, m.p. 178–179°, in 3 ml. of methanol containing one drop of concentrated sulfuric acid was refluxed on a boiling water bath for 20 minutes. The resultant yellow solution was diluted with dilute sodium bicarbonate solution and the crystalline precipitate which was separated on standing, was collected, washed with water and dried. Crystallization of the dried precipitate from methanol afforded pale yellow prismatic needles of m.p. 183–184°, $\lambda_{max.}^{alc.}$ 284 mμ (log ε 4.42). The infrared spectrum of this ester showed the following absorption bands (in Nujol): hydroxyl, 2.85 μ; ester, 5.82 μ; $\Delta^{4,6}$-3-ketone, 6.01, 6.19 and 6.34 μ. The sample was not sufficiently pure to give satisfactory analytical data, but the identity with an authentic sample of methyl 12α-hydroxy-3-keto-$\Delta^{4,6}$-choladienate (*3*) with m.p. 184–185.5° was proved by mixed melting point determination and infrared comparison.

Analysis Calcd. for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06.
Found: C, 74.37; H, 9.67.

DISCUSSION

A metabolic pathway of the breakdown of cholic acid by *Streptomyces* species has been presented in a previous paper (*4*), where 7α-hydroxy-3,12-diketo-Δ^4-cholenic acid was presumed to be a direct precursor of 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid.

However, the report of Eguchi (*1*) and this investigation have led to a suggestion of an alternate pathway in cholic acid cleavage by microorganisms; *i.e.* as discussed previously (*4*, *5*), if the Δ^4-3-ketone group is necessary for side chain cleavage of cholic acid (I), 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid (III) may be considered to be a direct

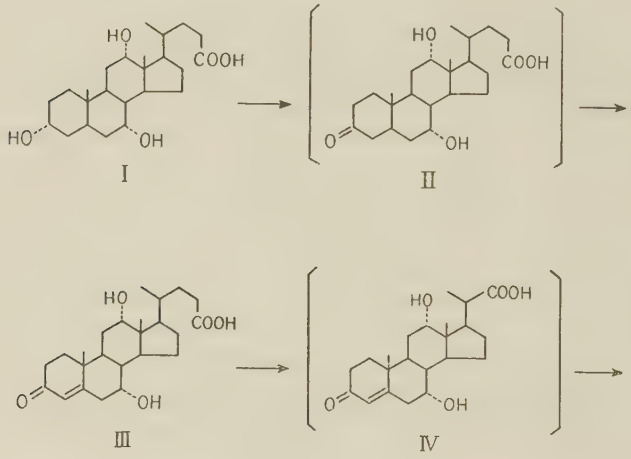
substrate of a bacterial enzyme which catalyses β-oxidation of side chain, though the acid (II) and the C_{22} acid (IV) has not been isolated in this case.

Thus in the case of bacteria (strain CE-1 and CF-1) the cholate cleavage may have a different pathway (Scheme 1) as compared with *Streptomyces spp.*

Further confirmation of the structure of the cholic acid derivative isolated in this experiment, and the isolation of its oxidized product (C_{22} acid) are now under investigation.

SCHEME 1

Proposed Degradation Pathway of Cholic Acid by Bacteria CE-1 and CF-1



SUMMARY

1. A Gram-negative bacterium (strain CF-1) which can utilize cholic acid as the sole source of carbon for growth was isolated from soil.

2. One of the degradation products of cholic acid by the strain CF-1 was isolated as crystals and it was demonstrated that the newly isolated acid is probably 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid.

3. A possible pathway for the degradation of cholic acid by the strain CF-1 was discussed.

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A PHOSPHORYLATIVE PROCESS, ACCOMPANIED BY PHOTOCHEMICAL LIBERATION OF OXYGEN, OCCURRING AT THE STAGE OF NUCLEAR DIVISION IN CHLORELLA CELLS. II

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Using the technique of synchronous culture of *Chlorella*, we have shown that the algal cells perform a special type of phosphate assimilation at the stage immediately prior to the process of nuclear division (*1*). When the cells at earlier stages of " light cells " (L_1 or L_2)** were illuminated in the presence of phosphate, they consumed a considerable amount of phosphate with simultaneous evolution of oxygen. The phosphorus fixed was found, in the main, in an energy-rich form, which, by several qualitative reactions, was concluded to be a form of metaphosphate (or polyphosphate). Recently, the occurrence of a similar substance(s) in *Chlorella* cells was also reported by some other workers (*2, 3, 4*). The work that is reported below was performed with a view to get further information as to the mode of formation and metabolism of the phosphorous substance in algal cells.

METHODS

Chlorella ellipsoidea was grown synchronously—starting from " active dark cells "**—by the method reported previously from our laboratory (*5, 6*); culture temperature was 15°, illumination 10 kilolux, and the culture medium had the following composition; KNO_3 5.0 g., $MgSO_4 \cdot 7H_2O$ 2.5 g., KH_2PO_4 1.25 g., $FeSO_4 \cdot 7H_2O$ 0.003 g., Arnon's " A5 " solution 1 ml. (*8*). The synchrony of growth and the transition of developmental stages were checked by measuring the cell number and the packed volume of cells in the culture. At various stages of development, cell samples were taken out, and after being washed twice with distilled water, they were analysed for contents in total phosphorus (by Allen's method (*9*)), and the phosphorus in four fractions according to

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** For the definition of " light " and " dark " cells as well as the symbols, L_1, L_2, *etc.*, see our previous publications (*5, 6, 7*).

Schneider (*10*), namely, " acid-soluble," " alcohol-ether-soluble," " acid-insoluble " and " residual " freactions. The method of fractionation was the same as that reported previously (*1*). Combined with fractionating experiments, tracer experiments were carried out using radioactive phosphate which was added to the cell suspension in the amount of 10,000 to 15,000 c.p.m. per ml. of cell suspension.

RESULTS

During the course of the life cycle of algal cells, the phosphorus in the four fractions mentioned above showed changes in quantity as illustrated in Fig. 1. In this figure the contents of phosphorus are given both in percent of dry weight and in microgram per cell. The meaning of the symbols given for successive stages of cell development is as follows (*cf.* (*11*) :

D_n: " nascent dark cells " which are the young cells newly produced from mother light cells,

D_a: " active dark cells," the most chlorophyll-rich and photosynthetically active cells which are derived from D_n when illuminated,

D∼L: cells of transient stage between dark and light cells,

L_1: immature light cells which are large in size, but not yet ripe enough to perform cell-division when incubated in the dark,

L_2: half-mature light cells which can only partially divide when kept in the dark,

L_3: mature light cells which can completely divide when incubated in the dark,

L_4: fully ripened light cells which are at the stage immediately prior to the process of cell division.

It is apparent from the figure that the most striking P-assimilation occurred at the stage between D∼L and L_3. The main part of the phosphorus assimilated at this stage was in the acid-insoluble fraction, hydrolysable by 7-minute treatment with 1 *N* HCl at 100°, and precipitated with barium at pH 3–4, the characters indicating the presence of metaphosphate. Both total P and the phosphorus in the acid-insoluble fraction attained the highest level at L_3. Further ripening of L_3 (occurring in the dark) was accompanied by a marked decrease of acid-insoluble phosphorus and a slight increase of residual phosphorus which is, conceivably in the main, the phosphorus of phospho-proteins.***

*** On treating the residual fraction with 6 *N* HCl at 37° for 3 days, all the phosphorus contained in the fraction became acid-soluble. After removing HCl by evaporation at reduced pressure, the hydrolysate was subjected to paper chromatography

To follow the fates and mutual relations of phosphorous compounds in different fractions more in detail, the following experiments were performed. From the synchronous culture starting from D_a-cells, a cell sample was taken out at the stage of L_1 where the most pronounced P-assimilation used to take place. The cells were suspended in a medium containing labeled phosphate and left to assimilate the phosphate in the light. After 2 hours of assimilation, the cells were collected by centrifu-

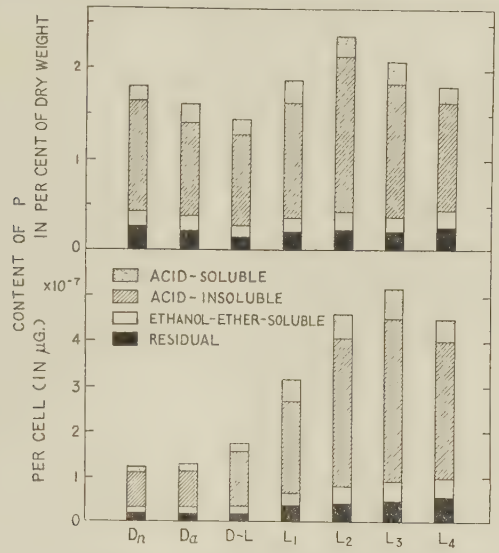

FIG. 1. Phosphorus content in various fractions as found in algal cells of different developmental stages. For the symbols of developmental stages see the text.

gation, washed once with ordinary culture solution (containing no P^{32}), suspended in the same solution, and then either (1) kept illuminated for further 6 hours (by the time of which the cells attained the stage of L_3), or (2) kept in the dark for 12 hours (under aerobic condition). In both cases no increase of cell number occurred during the incubation, the essential difference being that an increase of cell mass occurred in (1) but not in (2). Before and after the subsequent light- or dark-incubation (in the absence of exogenous P^{32}), the distribution of P^{32} in

using a mixture of butanol, acetic acid and water as a developer. Most part of phosphorus was found near the original point where the ninhydrin reaction was also distinctly positive.

various fractions of cell material was investigated. The results obtained are presented in Tables I and II.****

It appears from the data given in both tables that immediately after 2 hours' assimilation of P^{32} in the light, the radioactivity was found to be highest in the acid-soluble fraction, somewhat lower in the acid-insoluble fraction, and markedly lower in alcohol-ether-soluble and residual fractions. The fact that the level of P^{32} was higher in the acid-soluble fraction than in the acid-insoluble fraction appears to be in

TABLE I

Distribution in Various Fractions of Radioactive Phosphorus Which Had Been Assimilated by "Immature Light Cells," and Its Change Caused by the Incubation of Cells in the Light after Substitution of Exogenous P^{32} with P

Pre-assimilation of P^{32} lasted 2 hours in the light. The figures in parentheses indicate the radioactivity of acid-insoluble fraction which was obtained after the cells had been extracted with water of pH 9. See the text.

Fractions	Relative P^{32}-activity (in c.p.m. per 1×10^6 cells) found in each fraction when the P^{32}-assimilated cells were incubated in ordinary medium for	
	0 hour	6 hour
Acid-soluble	4356	564
Alcohol-ether-soluble	726	832
Acid-insoluble	2876 (640)	5084 (730)
Residual	470	864
Total	8428	7344

contradictory to the findings illustrated in Fig. 1. It may, however, be interpreted as being due to the situation that the exchange of pre-existing P with P^{32} occurred more markedly in the substances contained in the acid-soluble fraction than in those contained in the acid-insoluble fraction. The figures in parentheses given in the fifth row of the tables indicate the radioactivity of acid-insoluble fraction which was obtained after the cells had been extracted with water of pH 9. The rest of the

**** The two sets of experiment were performed using different cell samples.

activity, which forms the major part of the total activity of the acid-insoluble fraction, may be assumed to be due to metaphosphate or related compounds.

When the P^{32}-assimilated cells were transferred to ordinary medium and incubated in the light for 6 hours, there occurred a striking decrease of P^{32} in the acid-soluble fraction and concomitantly a marked increase of P^{32} in the acid-insoluble fraction, indicating that a part of the former had been transferred to the latter. Although smaller in quantity, there

TABLE II

Distribution in Various Fractions of Radioactive Phosphorus which Had Been Assimilated by "Immature Light Cells," and Its Change Caused by the incubation of Cells in the Dark after Substitution of Exogenous P^{32} with P.

Pre-assimilation of P^{32} lasted 2 hours in the light. The figures in parentheses indicate the radioactivity of acid-insoluble fraction which was obtained after the cells had been extracted with water of pH 9.

Fractions	Relative P^{32}-activity (in c.p.m. per 1×10^6 cells) found in each fraction when the P^{32}-assimilated cells were incubated in ordinary medium for	
	0 hour	12 hours
Acid-soluble	895	220
Alcohol-ether-soluble	120	90
Acid-insoluble	739 (162)	474 (95)
Residual	234	320
Total	1988	1104

occurred also an increase of P^{32} in both alcohol-ether-soluble and residual fractions. It should be remarked that during the incubation in the light (after substitution of exogenous P^{32} with P), the total P^{32}-content in cells decreased to some extent owing to the exchange with exogenous P, whereas the total phosphorus content (P and P^{32}) was found to have increased by *ca.* 10 percent in the meantime. The decrease of P^{32} in the acid-soluble fraction might partially be due to its turn-over, which, as mentioned above, seems to occur most easily in the substances contained in the fraction.

Somewhat different phenomena were observed when P^{32}-assimilated cells were incubated in the dark after substitution of exogenous P^{32} with P (Table II). By chemical analysis of algal cells it was found that by the effect of dark incubation the phosphorus in the acid-insoluble fraction had decreased by *ca.* 15 percent, while the quantity of phosphorus in other fractions remained practically unchanged. In terms of radioactivity of P^{32}, on the other hand, a decrease was observed in all fractions except in the residual fraction, the most significant decrease occurring again in the acid-soluble fraction. Noteworthy is the fact that the P^{32} in the acid-insoluble fraction decreased during the dark incubation, while it increased considerably during the light incubation (Table I). This fact corresponds to the earlier observation (*1*) that the accumulation of metaphosphate in light cells occurred only in the light and not in the dark.

The data given in Tables I and II may be construed as suggesting: (1) that the photochemical incorporation of P in the acid-insoluble fraction occurs *via* a certain substance or substances (presumably ATP and/or related substances) contained in the acid-soluble fraction, and (2) that the P which had been incorporated in the acid-insoluble fraction is further consumed by a certain light-independent reaction or reactions, and probably transferred, at least partly, to the proteinous substances contained in the residual fraction.

SUMMARY

1. Using the technique of synchronous culture, the mode of formation and metabolism of metaphosphate (or polyphosphate) in *Chlorella* cells was investigated.

2. The most striking P-assimilation occurred at the stage between " dark cells " and " mature light cells " (according to the terminology used by Tamiya *et al.* (*5, 6, 11*)). The main part of the phosphorus assimilated at this stage was in the " acid-insoluble " fraction, hydrolysable by 7-minute treatment with 1 *N* HCl at 100°, and precipitated with barium at pH 3–4, indicating the presence of metaphosphate. Both total P and the phosphorus in the " acid-insoluble " fraction attained the highest level at " mature light cells." Further ripening of these cells was accompnied by a marked decrease of " acid-insoluble " phosphorus and a slight increase of " residual " phosphorus which is, conceivably in the main, the phosphorus of phospho-proteins.

3. To follow the fates of phosphorus in various fractions, the

" immature light cells," which had been fed with radioactive phosphate for 2 hours in the light, were transferred to ordinary culture medium (without P^{32}) and further incubated in the light or in the dark. Immediately after the assimilation of P^{32} in the light, the radioactivity was found to be highest in the acid-soluble fraction, somewhat lower in the acid-insoluble fraction, and markedly lower in alcohol-ether-soluble and residual fractions. It appears that the exchange of pre-existing P with P^{32} occurs most markedly in the substances contained in the acid-soluble fraction.

4. When the P^{32}-assimilated cells were transferred to ordinary medium and incubated in the light for 6 hours, there occurred a striking decrease of P^{32} in the acid-soluble fraction and concomitantly a marked increase of P^{32} in the acid-insoluble fraction, indicating that the former had been partially transferred to the latter. Although smaller in quantity, there occurred also an increase of P^{32} in both alcohol-ether-soluble and residual fractions.

5. When the P^{32}-assimilated cells were incubated in the dark after substitution of exogenous P^{32} with P, a decrease of P^{32} was observed in all fractions except in the residual fraction, the most significant decrease occurring again in the acid-soluble fraction.

6. Based on various observations mentioned above, it was inferred that (1) the photochemical incorporation of P in the acid-insoluble fraction occurs, most probably via a certain substance or substances—presumably ATP and/or related substances—which are contained in the acid-soluble fraction, and (2) that the phosphorus which had been incorporated in the acid-insoluble fraction is further consumed by a certain light-independent reaction or reactions, and probably transferred, at least partly, to the proteinous substances contained in the residual fraction.

This work was carried out as a part of the studies on the growth of *Chlorella* directed by Prof. H. Tamiya, to whom the writer is indebted for his guidance. Thanks are due to the Ministry of Education and the Rockefeller Foundation for grants in aid of this work.

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METABOLIC PRODUCTION OF GLUCOSAMINIC ACID

Sirs:

The study of the metabolism of D-glucosamine has recently made a significant progress and the existence of anaerobic steps described below has been verified (*1–5*).

Glucosamine → Glucosamine-6-phosphate → Fructose-6-phosphate + NH_3

The author found in the study of the aerobic degradation pathways that *Pseudomonas fluorescence* oxidized glucosamine into glucosaminic acid. This is probably the first case in which glucosaminic acid appears in metabolic patterns.

P. fluorescence consumes only one atom of oxygen per mole of glucosamine degraded and the reaction mixture shows dropped pH and reduces Somogyi reagent remarkably in spite of almost negative Elson-Morgan reaction. Paperchromatography reveals the formation of glucosaminic acid (R_f=0.35, *tert.* butanol-formic acid-water 3:1:1).

Table I represents the oxygen uptake by *P. fluorescence* when several sugars are used as substrates.

TABLE I

Substrates	Atoms of oxygen-uptake per mole of each substrate (30°, pH 7.0)
Glucosamine	1
Glucose	7
Fructose	7–8
Gluconic acid	6

Using 430 mg. (2 μM) of glucosamine hydrochloride as the substrate 180 mg. of crystalline glucosaminic acid was isolated and identified paperchromatographically. Nitrogen analysis of the product showed good coincidence with the theoretical value ($C_6H_{13}O_6N$, found: 7.12 per cent, theoretical: 7.18 per cent).

A cell-free extract was obtained by disintegrating the bacterial cells with sillica sand and centrifuging at 20,000 r.p.m.

Table II indicates the activity of the enzyme preparation and intact cells.

TABLE II

	Glucosamine added (μM)	Oxygen consumed (μM)	Glucosamine disappearing (μM)	Glucosaminic acid formed (μM)
Cell-free extract	8.0	3.6	7.3	7.2
Intact cells	8.0	4.0	7.8	8.2

Both intact cells and the enzyme preparation shoed strong catalase activity.

It is an unexpected fact that glucosaminic acid which has almost no reducing action against Fehling reagent reduces Somogyi reagent remarkably.

Details of the work will be published elswhere and the work will be continued.

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